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A New Spectrophotometric Method for The Determination of Macitentan in Human Plasma

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Abstract

Macitentan is an endothelin receptor antagonist drug used in the treatment of pulmonary arterial hypertension (PAH). PAH is defined as a serious disease of the arteries (pulmonary arteries) that connect the lungs to the heart. The aim of our study is to develop and validate a new, sensitive, simple, accurate and reliable UV spectrophotometric method for the quantification of macitentan in human plasma. Using a wavelength of 269 nm, macitentan was quantitatively analyzed. For standard solution concentrations of 1–30 µg/mL and for human plasma concentrations of 2–30 µg/mL, respectively, the method was linear. By computing the correlation coefficient, linearity was determined. In standard solution and human plasma, these values were found to be 0.9996 and 0.9991, respectively. In terms of accuracy, sensitivity, precision, and repeatability, the developed spectrophotometric method was deemed satisfactory. In addition to this, this method could be directly and easily applied to both bulk and human plasma.

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1. INTRODUCTION

Pulmonary hypertension (PH) is a life-threatening cardiopulmonary disease associated with progressive elevation of pulmonary pressure (Zolty, 2020). Pulmonary arterial hypertension (PAH) is a rapidly progressive and fatal subgroup of PH. It is characterized by endothelial dysfunction, intima, and smooth muscle cell proliferation. Progressive increases in pulmonary vascular resistance and pressure decrease right ventricular performance and then result in right heart failure and death (Montani *et al*., 2013). An effective treatment for PAH is endothelin receptor antagonist macitentan (Bruderer *et al*., 2012). Macitentan, a dual endothelin-receptor antagonist, was developed by altering the structure of the medication bosentan. Macitentan is a medication that greatly lowers morbidity and mortality in individuals with pulmonary arterial hypertension and is distinguished by sustained receptor binding and improved tissue penetration (Pulido *et al*., 2013). The chemical formula for macitentan is C19H20Br2N6O4S and the chemical name is N-[5-(4-bromophenyl)-6-{2-[(5-bromo-2-pyrimidinyl)oxy] ethoxy}-4-pyrimidinyl]-N′-propylsulfamide (Figure 1).

In the context of intensive attempts to optimize blockage of the ET axis while producing drugs with superior adverse-effect profiles compared to earlier compounds in the class, macitentan is a dual ET-receptor antagonist (ERA). Macitentan belongs to the sulfamide class of chemicals, unlike bosentan and sitaxsentan, which both have a sulfonamide structure (Dingemanse *et al*., 2014; Trow *et al*., 2014). It has been proven that macitentan has much more potent antagonism than bosentan or ambrisentan since its functional inhibition constant (KB)

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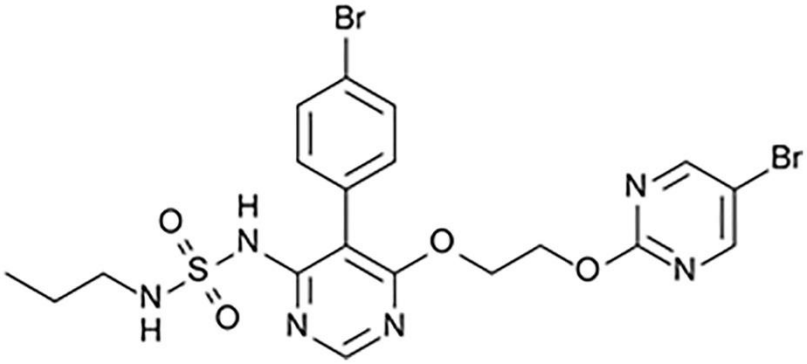
ratio of 6.3 is larger than those of both drugs (0.7 and 1.0, respectively) (Gatfield *et al*., 2012). Averagely 8 hours after taking the medicine, the blood concentration reaches its peak. The drug has a bioavailability of roughly 74% (de Kanter *et al*., 2016; Albayrak and Atila, 2019).

Figure 1. The chemical structure of macitentan.



When the literature is examined, there are methods developed using HPLC and LC-MS/MS for the analysis of macitentan from commercial preparations or plasma (Yu *et al*., 2015; Lakshmi *et al*., 2016; Enderle *et al*., 2017; Thummar *et al*., 2018; Albayrak and Atila, 2019; Albayrak *et al*., 2019). Utilizing modern analytical techniques for drug assays has a number of benefits, including simplicity, speed, good specificity, excellent precision, and accuracy. However, they require sophisticated tools that might not be present in most laboratories (Atila, 2012). The objective of the current work is to develop a fast, sensitive, and precise spectrophotometric method for estimating macitentan levels in human plasma. In accordance with the standards of the International Conference on Harmonization (ICH) (Riley and Rosanske, 1996), the developed method was validated.

2. MATERIAL and METHODS

2.1. Chemicals and Reagents

The Atatürk University Research Hospital in Erzurum, Turkey, provided the plasma samples. Santa Cruz Biotechnology supplied the macitentan in its purest form, which was bought. We bought methanol (purity>99%) and acetonitrile (purity>99%) from Merck in Germany. Acetic acid and HCl of analytical grade were purchased from Sigma-Aldrich in Germany. The ultrapure water equipment was used to obtain the water that was used to prepare the mobile phase (Merck Millipore Simplicity-UV, Darmstadt, Germany). The vacuum concentrator (Eppendorf Concentrator Plus) was supplied from Hamburg, Germany.

2.2. Instrumentation

Thermo Scientific MultiSkan Go model UV-VIS spectrophotometer was used for all spectrophotometric measurements. A quartz cuvette was used in the scan (200 - 400 nm) to determine the wavelength that gives the maximum absorbance. 96-well plates were used for all other measurements.

2.3. Wavelength Selection

Using the UV/Visible spectrophotometer's SCAN mode, standard macitentan solutions (100 µg/mL) were scanned in the range of 200 nm to 400 nm against a solvent blank to obtain a normal (zero order) spectrum.

2.4. Preparation of the Standard, Plasma and Quality Control Solutions

Weighed 1 mg of pure macitentan was transferred to a 100 ml flask and dissolved in a mixture of methanol:water (20:80, v/v) used as a solvent. It was then vortexed for 3 min. The

required dilutions from stock solution were performed in order to prepare the 8 standard solutions (1, 2.5, 5, 10, 15, 20, 25 and 30 µg/mL) in solvent (methanol:water (25:75, v/v)). From the stock standard solution, three quality control (QC) samples with concentrations of 4, 17.5, and 27.5 µg/mL were prepared. To achieve final concentrations between 2 and 30 µg/mL (2, 3, 5, 10, 15, 20, 25, and 30 µg/mL) and QC samples at three distinct levels (4, 17.5, and 27.5 µg/mL), different amounts of macitentan were spiked into plasma (0.5 mL). A previously described method for the extraction of macitentan from plasma samples was slightly modified (Albayrak *et al*., 2019). 500 µL of methanol was added to the plasma sample and vortexed for 1 min. Then 1 ml of 1M HCl was added and vortexed for 1 min. Diethyl ether was added in a 5 mL aliquot, which was vortexed for 1 minute. Centrifuging the mixture took place at 4°C for 8 minutes at 3500 rpm. In a vacuum concentrator, the supernatant was evaporated. The remaining material was then dissolved in 1 mL of solvent.

3. RESULTS and DISCUSSION

3.1. Linearity

Six samples were made for each concentration of the macitentan standard solution, and they were all measured at 269 nm. In the concentration range of 1–30 µg/mL for the standard solution (Figure 2) and 2–30 µg/mL for human plasma (Figure 3), a calibration curve was linear. Human plasma and the standard solution had linearity correlation values of 0.9991 and 0.9996, respectively. For the suggested method, the least squares regression method was used to produce the regression equations for slope (Sb) and intercept (Sa). Table 1 presents the findings.

Figure 2. Calibration curve for standard solutions

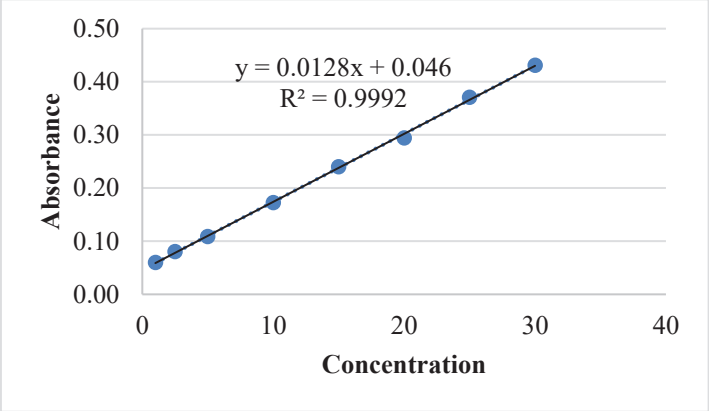


Figure 3. Calibration curve for plasma solutions

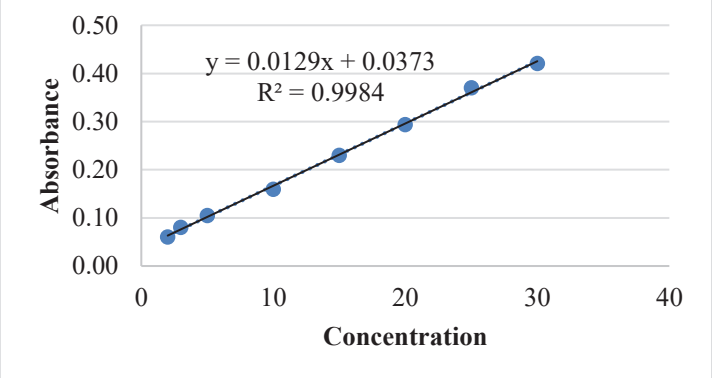


Table 1. Regression analysis of macitentan

Method	Standard	Plasma
Range (µg/mL)	1–30	2–30
LR[a]	0.0128x + 0.046	0.0129x + 0.0373
Sa	$1.6x10^{-3}$	$2.4x10^{-3}$
Sb	$2.9x10^{-2}$	$4.1x10^{-3}$
R	0.9996	0.9991

LR: linear regression, Sa: Standard deviation of intercept of regression line Sb: Standard deviation of slope of regression line, R: Coefficient of correlation

3.2. Linearity

A series of solutions at concentrations that have a smaller value than the smallest value of calibration curves, were prepared and their absorbances were monitored by six times. Their %RSD were determined. It was stated that the concentration value whose %RSD values are lower than 20% was LOD and LOQ was defined as %RSD value is lower than 10%. For the spectrophotometric method, LOD values were found as 0.8 µg/mL for standard solution and 1.4 µg/mL for plasma solution. LOQ values of 1 µg/mL for the standard solution and 2 µg/mL for the plasma sample were determined.

3.3. Precision and Accuracy

Table 2. Precision and accuracy of proposed method for determination of macitentan.

Material	Added (µg/mL)	Intra-day				Inter-day			
		Found (µg/mL)	±SD	Accuracy	Precision RSD%	Found (µg/mL)	±SD	Accuracy	Precision RSD%
Standard	4	4.09	0.19	2.25	4.65	4.13	0.19	3.25	4.60
	17.5	17.73	0.92	1.31	5.19	17.95	0.92	2.57	5.13
	27.5	28.01	1.58	1.85	5.64	27.85	1.58	1.27	5.67
Plasma	4	4.17	0.23	4.25	5.52	4.19	0.23	4.75	5.49
	17.5	17.11	1.32	-2.23	7.71	17.03	1.32	-2.69	7.75
	27.5	28.21	1.78	2.58	6.31	28.39	1.78	3.24	6.27

Accuracy and precision values were calculated for both standard solutions and plasma solutions. Simply put, the statistical point of view for the outcomes of repeated independent tests using a suggested method is how precision in drug analysis is defined. The degree to which the outcome resembles the real quantity is referred to as accuracy (Senol, 2018). Quality control solutions were prepared at 3 different concentrations (4, 17.5, and 27.5 µg/mL) in macitentan calibration curve. Intra-day (six times in a day with the same methods and conditions) and inter day (6 times in different 3 days with the same method) analyses of these solutions were performed. Average of the analysis results and standard deviation were determined. The accuracy was given by relative error (RE = (found-added)/added) x 100) and the precision by relative standard deviation (%RSD = (SD/average) x 100). The results are given in Table 2.

3.4. Specificity and Selectivity

No interference was detected while performing the analysis on a blank solution, which was monitored between 200 and 400 nm. Excipients were also dissolved in a methanol and water mixture and the suggested method was used on them. There is no overlapping absorbance recorded throughout the analysis. As a result, it is clear that the method is specific for determining macitentan in plasma and bulk. Figure 4 and Figure 5 display the spectra of standard solutions and plasma at various concentrations.

Figure 4. Spectra of standard macitentan solutions

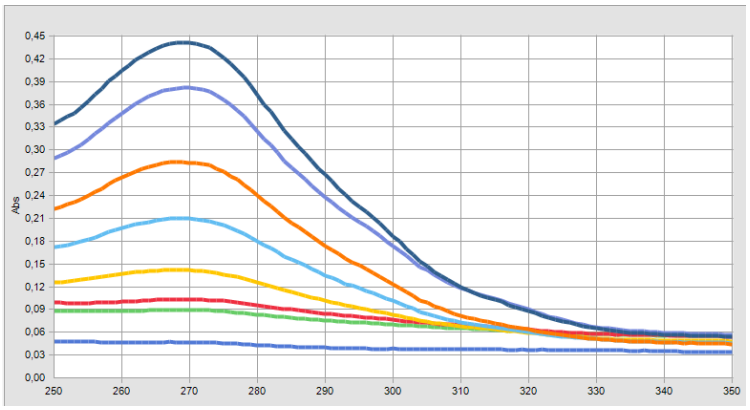
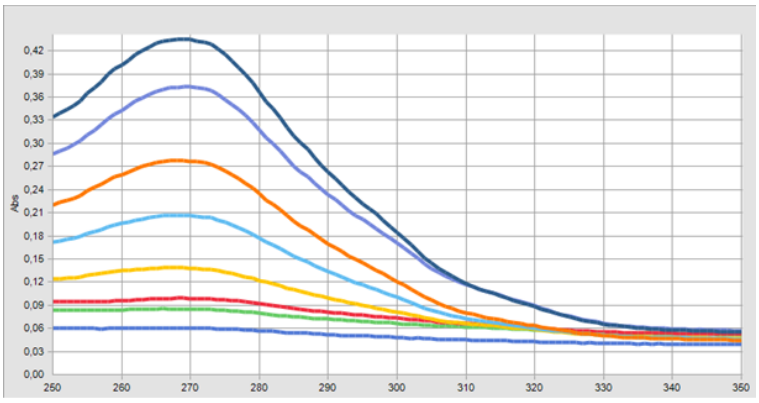


Figure 5. Spectra of plasma macitentan solutions



3.5. Analytical Recovery

In the plasma recovery study, three different concentrations of macitentan quality control solutions (4, 17.5, and 27.5 µg/mL) were spiked into the plasma, mixed and extracted by the extraction method. The solutions obtained after the extraction were measured and the percentage recovery values were determined by proportioning the values with the values obtained from the standard working solutions. The obtained recovery values of human plasma were found to be 104.3%, 97.8% and 104.5%.

The developed method was found to have a short analysis time, a high sensitivity, a high recovery rate, and a high correlation coefficient. Another instance that emphasizes our work is the liquid-liquid extraction technique we employ. We employed a liquid-liquid phase extraction technique that does not require numerous steps, drawn-out procedures, or expensive ingredients.

4. CONCLUSION

This study developed and validated a UV-Vis spectrophotometric method for the quantitative assessment of macitentan in plasma and bulk samples. By using this method, simple derivatization procedures are not required for the quantitative determination of macitentan in plasma. To the best of our knowledge, this is the first study based on the spectrophotometric measurement of macitentan. According to ICH, the method's sensitivity, specificity, linearity, accuracy, and precision were used to validate it. The analytical method is applicable to both clinical laboratories and routine analysis.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

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Biological Evaluation of Aminoindane Derivatives as Antibacterial Agents

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Abstract

The aminoindane ring is the most studied skeletal structure in synthetic chemistry, in the synthesis of intermediates and because it is a biologically active molecule. Bioactive properties of the aminoindane ring include antibacterial, antiviral, antiapoptotic, antipyretic, analgesic, anticonvulsant, and antiparkinsonian activities. The most important bacteria in responsibility of nosocomial infections obtained in hospitals across the world are *Acinetobacter baumannii* and Methicillin-resistant *Staphylococcus aureus*. MRSA and *A. baumannii* have become resistant to numerous antibiotics. The development of novel antibiotic drugs is thus urgently needed. This study as investigated at the antibacterial effects of previously synthsized compounds **8** and **9** on *A. baumannii* and MRSA disease microorganisms. Studies on antibacterial activity employed disc diffusion and microdilution techniques. The investigation revealed that whereas the pathogenic organisms of compounds **8** and **9** formed zones that inhibited bacterial development, no zones formed in compounds **5**, **6**, and **7**. Compounds **8** and **9**'s zone diameters against *A. baumannii* and MRSA were estimated to be between 0.7 and 1.0 mm. The range of MIC values was 3.9025–15.625 µg/ml. These results was envisaged the conclusion that derivatives with an aminoindane ring may function as MRSA and *A. baumannii* antibacterial agents.

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1. INTRODUCTION

Aminoindanes are compounds that have a five-membered cyclopentane ring with an amino group and a six-member benzene ring. The pharmacophore features of aminoindane derivatives make them relevant in the realm of medicinal chemistry even though they are often utilized as intermediates in the production of several physiologically active chemicals (Sainsbury *et al*., 2011). The pharmacological effects of aminoindane derivatives include antibacterial (Osowole *et al*., 2012), antiviral (Ugliarolo *et al*., 2012), antiapoptotic (Thiry et al., 2006), antipyretic (Barlow and Walsh, 2008), analgesic (Barlow and Walsh, 2010), anticonvulsant (Chazalette *et al*., 2004), and antiparkinson (Bertolini *et al*., 1992) activity. For example, *5-methoxy-2-aminoindane* **(1)**, *5-methoxy-6-methyl-2-aminoindane* **(2)**, *5,6-methylenedioxy-2-aminoindane* **(3)** and *N-methyl-2-aminoindane* Psychoactive drugs such as aminoindane **(4)** are used commercially (Coppola and Mondola, 2013) (Figure 1).

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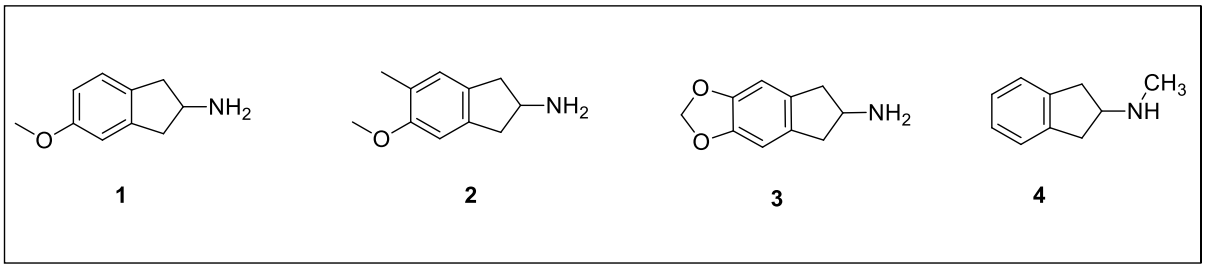
Figure 1. Some aminoindane drugs **1-4**.

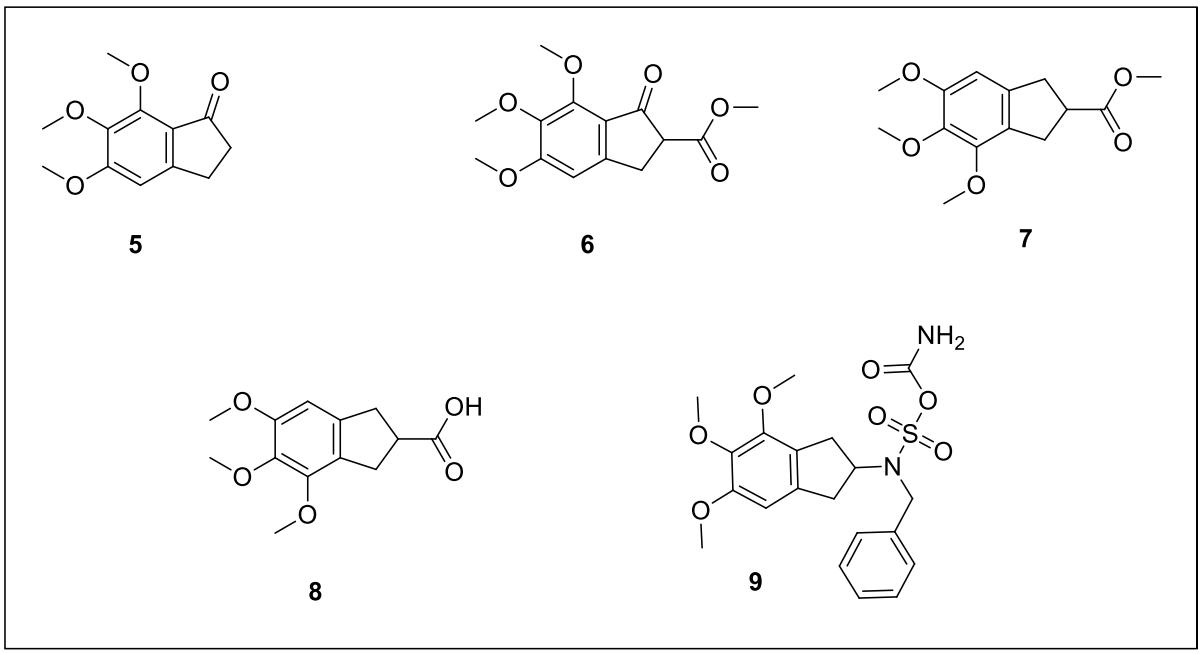


The most often isolated bacterium as a nosocomial infectious agent is *A. baumannii*, which is also antibiotic-resistant. *A. baumannii* is a non-motile, Gram-negative, non-fermenting, catalase-positive, oxidase-negative coccobacillus that is frequently identified in the hospital setting and has gained importance recently (Pompilio *et al*., 2021). Treatment for *A. baumannii* is challenging due to the organism's multidrug resistance (Kyriakidis *et al*., 2021).

S. aureus is a bacterial organism that causes serious infections. Particularly MRSA isolates, which have numerous drug resistances, produce serious infections. The most common cause of sepsis is thought to be a *S. aureus* infection acquired in the community (Turner et al., 2019). Hospital acquired infections are most frequently caused by MRSA isolates. *S. aureus* is resistant to harsh environmental conditions including dryness, high temperatures, and excessive salt because of its sporeless reproduction, but this also raises the risk of infection spread and limits treatment choices (Cheung *et al*., 2021).

This research sought to investigate the antibacterial activity of the previously synthesized (Ozgeris et al., 2015) aminoindane derivatives *5,6,7-Trimethoxy-2,3-dihydro-1H-inden-1-on* **(5)**, *Methyl-5,6,7-trimethoxy-1-oxo-2,3-dihydro-1H-inden-2-carboxylate* **(6)**, *Methyl-4,5,6-trimethoxy-2,3-dihydro-1H-inden-2-carboxylate* **(7)**, *4,5,6-Trimethoxy-2,3-dihydro-1H-inden-2-carboxylic acid* **(8)** and *Benzyl-N-(4,5,6-trimethoxy-2,3-dihydro-1H-inden-2-yl)sulfamoyl carbamate* **(9)** (Figure 2). MRSA and *A. baumannii*, two nosocomial pathogenic pathogens, served as the test group for the compounds antibacterial activity.

Figure 2. Aminoindane derivatives



2. MATERIAL and METHODS

2.1. Chemical and Material

Dimethyl sulfoxide (DMSO) (Merck) was used as a negative control and dissolving compound. For antimicrobial analyzes, Luria Bertaini Agar (LBA) (Difco) Mueller Hinton Agar (MHA) (Oxoid) and Mueller Hinton Broth (MHB) (Biolife), Netilmicin disc (Bioanalyse) were used as positive controls.

2.2. Instrumentation

Thermo Scientific MultiSkan Go model UV-VIS spectrophotometer was used for all spectrophotometric measurements. A quartz cuvette was used in the scan (200 - 400 nm) to determine the wavelength that gives the maximum absorbance. 96-well plates were used for all other measurements.

2.3. Strains of Bacteria

A. baumannii (ATCC BAA-1605) and MRSA (ATCC 43300), pathogenic microorganisms obtained in the culture collection of Erzurum Technical University, were employed for antibacterial activity assays. After a 24-hour incubation at 37°C, microorganisms were cultured in Luria Bertani Agar (LBA) medium and employed in investigations.

2.2.1. Research on Microbes Utilizing Disc Diffusion Analysis

One of the most used techniques for antibacterial testing, the disk diffusion test, uses a bacterial inoculum that has been distributed into MHA medium in accordance with 0.5 McFarland's instructions. Adsorbed substances were prepared as 1000 µg/ml (1 µg active substance + 100 µl DMSO + 900 µl dH_2O) and placed in petri plates in three replications. Microorganisms' antibacterial sensitivity was assessed by measuring the zone diameters that developed after incubation for 24 hours at 37°C. Netilmicin was chosen as the study's preferred positive control since it provided for the evaluation of the activities of the synthesized compounds. 10% DMSO was utilized as a negative control since the synthesized compounds were dissolved in it. The zone diameters of the compounds whose activity was determined as a result of the disk diffusion test were evaluated (Özgeriş *et al*., 2021).

2.2.2. MIC-Based Antimicrobial Research

The Minimum Inhibition Concentration (MIC), also known as the lowest antimicrobial agent concentration that inhibits bacterial growth, was established using the microdilution technique after the activities of the synthesized compounds were assessed in vitro. In the procedure to be used for this, 100 µl of MHB and 100 µl of synthesized compounds were added to each well of the 96-well plates. By using the serial dilution technique, starting with the first well, the compound's concentration was decreased from 1000 µg/ml to 1.95 µg/ml. The lowest concentrations that stop bacteria from growing and don't have any visible turbidity were identified as MIC values after 100 µl of bacterial culture inoculum adjusted according to 0.5 McFarland was added. The turbidity formed in the wells after the 24-hour incubation period was read in the spectrophotometer (600 nm wavelength) (Gormez *et al*., 2015).

3. RESULTS and DISCUSSION

3.1. Antimicrobial Activity

3.1.1. Antibacterial Effect

By using the disk diffusion and microdilution methods, the antibacterial activity of aminoindane derivatives synthesized against *A. baumannii* and MRSA was evaluated. These investigations revealed no zone image that would prevent the bacterial development of **5**, **6**, and

7 compounds. However, it was discovered that compounds **8** and **9** prevented the growth of the MRSA and *A. baumannii* bacteria. Further zone diameters are measured (Table 1).

Table 1. Disc diffusion analysis results of aminoindane derivatives.

Bacteria Strain	(8)	(9)	Control**
A. baumannii ATCC BAA- 1605	0,9*	1,0*	1,2
MRSA ATCC 43300	0,8*	0.7*	1,0

* zone diameter (cm) ** Positive control: netilmicin

The minimal inhibitory concentration of the aminoindane compounds **8** and **9** that have antibacterial activity against MRSA and *A. baumannii* was established. The MIC concentration is detailed in Table 2.

Table 2. Disc diffusion analysis results of aminoindane derivatives.

Bacteria Strain	(8)	(9)
A. baumannii ATCC BAA-1605	3,9025*	3,9025*
MRSA ATCC 43300	15,625*	3,9025*

*The MIC concentration value was calculated as µg/ml.

The antibacterial action of compounds having an aminoindane ring is well reported in the literatüre (Osowole *et al*., 2012). It was found that aminoindane derivatives showed antibacterial activity and that the most effective compound's MIC value was 500 µg/ml in the study by Patel et al. (2010) that investigated at the antibacterial activity of synthetic aminoindane derivatives against Gram-positive and Gram-negative pathogens. Gram negative *A. baumannii* and Gram positive MRSA were shown to be resistant to the antibacterial activity of **8** and **9** aminoindane derivatives in our investigation. Additionally, the fact that the MIC values in the research were lower than those in the literature demonstrates the superior efficacy of the **8** and **9** compounds. However, the investigation reveals that none of the compounds of aminoindane are antimicrobial. Although they are compounds with the same skeleton structure, the source of this condition is assumed to be caused by the varying structures in their side groups. Because compound **9** contains a sulfamoyl carbamate (Güller *et al*., 2021) functional group and compound **8** has carboxylic acid (Yang *et al*., 1997; Ukhov *et al*., 2011) in the variable group. In terms of numerous biological functions, it is well-known in the literature that these functional groups support the skeletal structure (Radadia *et al*., 2005; Dubrovin *et al*., 2015; Jiang *et al*., 2019).

4. CONCLUSION

As a result, the antibacterial activity of derivatives of aminoindane was established. Only **8** and **9** compounds were shown to have low MIC values and activity against *A. baumannii* and MRSA pathogenic microorganisms. These results contribute to the prediction that the compounds **8** and **9** could have therapeutic effects against *A. baumannii* and MRSA infections.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Damla Ruzgar: Studied antimicrobial activity. **Yusuf Akbaba:** Synthesized target products. **Bunyamin Ozgeris:** Synthesized target products and wrote the manuscript. **Arzu Gormez:** Designed the activity studies.

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Nepeta italica L. subsp. *cadmea* (Boiss.) A. L. Budantsev Oil Has a Pro-Apoptotic Effect on Saos-2 Cell Line

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Abstract

Nepeta sp. are used diuretic, anti-spasmodic, antiasthmatic, expectorant, cough suppressant, sedative, antiseptic, antipyretic, antiallergy and as a muscle relaxant. Besides, it also has many biological activities such as antioxidant, antibacterial, antiinflammatory, anticancer, antiparasitic, antiplatelet, antiangiogenesis, antimicrobial, antiviral, antifungal, insecticide, cytotoxic, phytotoxic, genotoxic, and apoptotic activities. Osteosarcoma is the most common primary bone cancer in children and adolescents, and its diagnosis is difficult due to the known heterogeneous nature of its pathogenesis. Chemotherapy for osteosarcoma is far from satisfactory, especially because of the presence of metastases or poor response to combination chemotherapy. Various studies have been conducted to evaluate the anti-apoptotic, anticancer, and cytotoxic potentials of different essential oil extracts from different species of the *Nepeta*. This study was carried out to confirm the cytotoxic and apoptotic effect of essential oil obtained by hydrodistillation from the aerial parts (flower, leaf, seed and stem) of *Nepeta italica* subsp *cadmea.* To figure out apoptotic properties of *N. italica* subsp *cadmea*. MTT assay, qRT-PCR with Bcl-2, caspase-9, Caspase-3, caspase-8, caspase-7, caspase-10, Bax gene primer and TUNEL assay accomplished. IC_{50} dose of *N. italica* subsp. *cadmea* oil was 10 µg/ml at 24 hours. Expression analysis showed that caspase8, caspase 10 were upregulated while Bcl-2 was downregulated. TUNEL assay results show the similar number of apoptotic cells in the control group then the *Nepeta* group. Therefore, it showed pro-apoptotic activity in conjunction with the expression profile.

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1. INTRODUCTION

Nepeta L. is the largest genus of the *Lamiaceae* family (Goldansaz *et al*., 2019; Sharma *et al*., 2021). There are about 300 species of this genus, most of them are aromatic (Acar *et al*., 2011; Formisano *et al*., 2011). It is represented by 39 species, 19 of which are endemic with a total of 50 taxa (Yılmaz *et al*., 2020). It spreads throughout the world, generally includes perennial plants (Sarikurkcu *et al*., 2019). The main diversity center of the genus *Nepeta* is Southwest Asia and the Western Himalayas (Acar *et al*., 2011).

Nepeta are used diuretic, anti-spasmodic, antiasthmatic (Goldansaz *et al*., 2019; Kaska *et al*., 2019), expectorant, cough suppressant (Sharma *et al*., 2021), sedative, antiseptic (Sarikurkcu et al., 2019), antipyretic, antiallergy (Ashrafi *et al*., 2020) and as a muscle relaxant (Akdeniz *et al*., 2020). Besides, it also has many biological activities such as antioxidant (Azizian *et al*., 2021), antibacterial, antiinflammatory (Kaska *et al*., 2019; Sitarek *et al*., 2020), anticancer, antiparasitic (Ashrafi *et al*., 2020), antiplatelet, antiangiogenesis (Al-Sheddi *et al*.,

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2018), antimicrobial, antiviral (Sharma and Cannoo, 2013; Afshar *et al*., 2017), antifungal, insecticide (Süntar *et al*., 2018; Sharma *et al*., 2021), cytotoxic, phytotoxic (Formisano *et al*., 2011), genotoxic, apoptotic (Süntar *et al*., 2018), antitumor, larvicide, analgesic, antidepressant and anticonvulsant (Süntar *et al*., 2018; Akdeniz *et al*., 2020).

The phytochemical compositions of the species belonging to the genus *Nepeta* particularly include nepetalactone isomers (Azizian *et al*., 2021), terpenes, phenolic acids, flavonoids and steroids (Akdeniz *et al*., 2020; Sharma *et al*., 2021). Essential oils obtained from these plants are very valuable due to their pleasant smell (Sarikurkcu *et al*., 2019) and are rich in monoterpenes (Formisano *et al*., 2011). More than thirty-five flavonoids from different species have been isolated from *Nepeta* species, including flavonols, flavones, flavanols, and flavanones (Sharma *et al*., 2021).

The medicinal effects of *Nepeta* species are generally due to their flavonoids and essential oils. The majority of *Nepeta* species contain lipophilic flavonoids of the flavone group on the surface of their leaves, and there are effective components of these plants that show their therapeutic effects due to their free radical scavenging and antioxidant capacities (Delshad *et al*., 2011).

Osteosarcoma is the most common primary bone cancer in children and adolescents, and its diagnosis is difficult due to the known heterogeneous nature of its pathogenesis. Osteosarcoma has been associated with cytogenetic and genetic abnormalities, such as activation of oncogenes and mutations in tumor suppressors (Kumar *et al*., 2016). Osteosarcoma arises in mesenchymal bone-forming cells, and it is one of the most common malignant tumors of the musculoskeletal system. Chemotherapy for osteosarcoma is far from satisfactory, especially because of the presence of metastases or poor response to combination chemotherapy (Anderson *et al*., 2010). The idea that natural molecules can inhibit cancer development was put forward more than 100 years ago, and the effects of small molecules extracted from fungi and plants on tumor development and growth in laboratories have begun to be studied. In this direction, studies have focused on the ability of these natural components to regulate signaling pathways and control basic cellular functions such as cell death, cell differentiation and cell proliferation (Amado *et al*., 2014). For this reason, natural products of plant origin have begun to attract more and more attention in recent years due to their anticancer functions (Han *et al*., 2015).

Essential oil extracted from various *Nepeta* species such as *N. menthoides* Boiss. & Buhse (Delshad and Parvizi., 2014), *N. cataria* L. (Emami *et al*., 2016), *N. deflersiana* Schweinf. ex Hedge (Al-Taweel *et al*., 2017), *N. gloeocephala* Rech. f. (Ashkezary *et al*., 2017) can be used in various human cancer cell lines were investigated for its apoptotic activity. At the same time, there is *N. deflersiana* (Al-Oqail *et al*., 2015; Al-Sheddi *et al*., 2018) anticancer and various studies have been conducted to evaluate the cytotoxic potential of *Nepeta* species such as *N. cataria* L. var. *citriodora* (Becker) Balb., *N. cataria* L. (Suschke *et al*., 2007), *N. menthoides* (Boiss & Buhse) (Kahkeshani *et al*., 2014), *N. ucrainica* L. *kopetdaghensis* (Shakeri *et al*., 2014), *N. sintenisii* Bornm (Shakeri *et al*., 2016), *N. binaloudensis* Jamzad (Afshar *et al*., 2017), *N. curvidens* Boiss. & Balansa (Ashrafi *et al*., 2020), *N. govaniana* (Wall. ex Benth.) Benth. (Dar *et al*., 2014), *N. heliotropifolia* Lam. and *N. congesta* Fisch. & C.A. Mey. subsp. *cryptantha* (Boiss. & Hausskn.) Dirmenci & Yıld (Akdeniz *et al*., 2020), *N. rtanjensis* Diklic & Milojevic, *N. sibirica* L. (Tsuruoka *et al*., 2012).

N. cadmea Boiss was reduced to a subspecies of *N. italica* by A.L. Budantsev and *N. italica* L. subsp. *cadmea* is arranged as. In addition, *Nepeta italica* L. subsp. *cadmea* is a synonym for *N. cadmea Boiss* (Dirmenci, 2003). The endemic species *N. cadmea Boiss.* is a herbaceous, perennial, generally aromatic plant rich in essential oil content (Yılmaz *et al*.,

2020). It is distributed in Western, Southern and Southwestern Anatolian regions of Turkey (Dirmenci, 2003).

We aimed to investigate the anticancer properties of *N. italica* L. subsp. *cadmea* oil in Saos-2 Osteosarcoma cell line through the apoptosis pathway in this study.

2. MATERIAL and METHODS

2.1. Plant Material and Essential Oil Preparation

N. italica subsp. *cadmea* samples were collected from Honaz Mountain in July-August. It was dried by monitoring continuously for 4 weeks in a dark, light and moisture-free environment. Then, the flowers, leaves, seeds and stems of the dried plants were cut into small pieces and ground into powder in a grinder. The plant extract was obtained by using the Clevenger apparatus with Hydrodistillation-HD, one of the distillation methods. The process was carried out by boiling the water and plant (powder) material in a glass balloon connected with the cooler for 4-6 hours, condensing the oil molecules moving with the water vapor in the cooler and separating them from the water.

2.2. Gas Chromatography of *Nepeta italica* subsp. *cadmea* Oil

Analysis of *Nepeta italica* subsp. *cadmea* essential oil (EO) was performed by gas chromatography (GC) using two types of detectors, flame ionization (FID) and mass-spectrometric (MS) by Bezmialem Vakıf University Phytotheraphy Education Research and Application Center (BITEM).

2.3. Cell Culture

For Saos-2 human osteosarcoma cell culture, DMEM-high glucose (Gibco) culture medium supported by 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco) was used. Saos-2 osteosarcoma cells were grown in 5% CO_2, 95% air humidified incubator at 37 °C under suitable cell culture conditions. The medium was removed, and fresh completed medium was added every 2-3 days. After Saos-2 cells reached 80% to 90% confluency in culture dishes, trypsinization was performed using 0.25% trypsin-EDTA (Gibco).

2.4. MTT Assay

The Effects of *N. italica* subsp. *cadmea* oil on cell proliferation in Saos-2 osteosarcoma cells were determined via MTT colorimetric-based assay according to manufacturer methodology (MTT Cell Viability Assay Kit; Biotium cat no: 30006). Saos-2 cells were seeded into 96-well plates at a concentration of 1×10^4 cells per well. Saos-2 cells were seeded in 96-well culture plates, 1×10^4 cells per well, and following their adherence, the cells were treated with different concentrations of *N. italica* subsp. *cadmea* essential oil in a dose range of 0.5 µg/ml and 10 µg/ml. Following incubation at doses treated for 24, 48, and 72 hours, the dose and time-dependent cytotoxic potential of the essential oil was investigated by MTT test. The essential oil was dissolved with ethanol and since the concentration of solvent ethanol was kept below one thousandth in applications, a working group was not formed for the solvent. Following the incubation, MTT solution was added, and reading was performed at 570 nm and 630 (reference wavelength) wavelengths with the help of a microplate reader (Biotek). Cell viability was determined in the groups treated the plant oil depending on the dose and time.

2.5. Analysis of Gene Expression by Real-Time PCR

Total RNA from the control group of Saos-2 cells and IC_{50} dose group (10 µg/ml Nepeta oil) of essential oil for 24 hour was isolated by using RNeasy Mini Kit (Qiagen Cat: 74104) according to the manufacturer's protocols. Complementary DNA (cDNA) was synthesized based on the total RNAs using the Quantitect Reverse Transcription Kit (Qiagen, 205311),

according to the manufacturer's protocol. mRNA expression changes of apoptosis related genes such as caspase-9, caspase-8, caspase-3, caspase-7, caspase-10, Bcl-2, Bax genes were detected in this study. Beta-actin was used as house-keeping gene for normalization of the PCR data. Real-time PCR tests were performed by Rotor Gene (Qiagen) according to the Quantitect SYBR Green PCR kit (Qiagen, 204143) protocol.

2.6. Apoptosis Detection

Effects of *Nepeta* oil on apoptosis in Saos-2 cells were determined by In Situ Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland. Saos-2 cells were plated per well onto Lab-Tek II Chamber Slides (Nalge Nunc International, Tokyo, Japan) as $3x10^4$ cells per plate. Sample slides of control and dose groups were incubated kit reaction mixture according to the manufacturer's protocols.

2.7. Statistical Analysis

All MTT, Tunel and RT-PCR assays were performed as triplicated. The mean ± standard error was evaluated by Microsoft Excel. RT-PCR data were analyzed with the $2^{-\Delta\Delta CT}$ method and quantitated with an online analysis platform (https://dataanalysis2.qiagen.com/pcr). RT2-Profiles™PCR Array Data Analysis program was used for fold calculation and statistical analysis. "Student t-test" was used for the comparison of gene expressions in the control and dose groups. ($p< 0.05$ was evaluated as significant statistically).

3. RESULTS and DISCUSSION

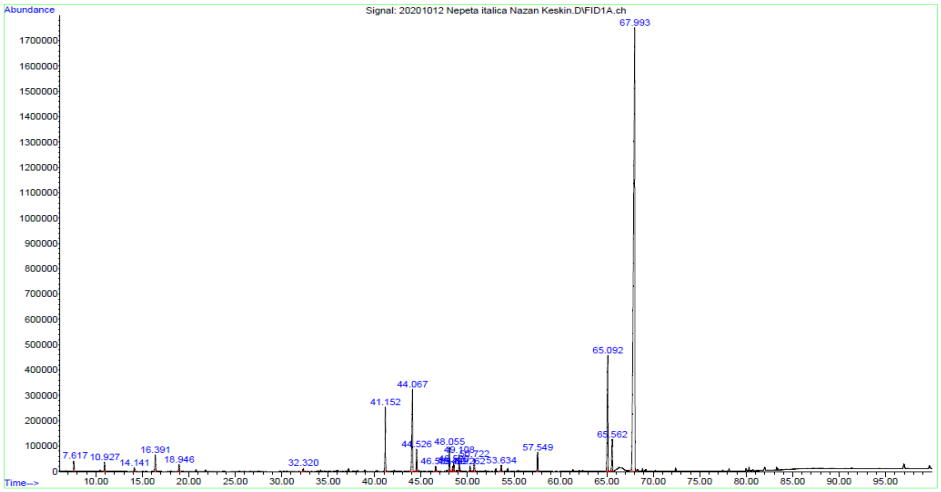
3.1. Chemical Composition

According to the GC-MS data (Table 1) and chromatogram (Figure 1), stereoisomers of nepetalactone, a monoterpene, constitutes the bulk of the essential oil composition (74%). Then the second most abundant substance was caryophyllene (6.89%). The other compounds were linalool (3.97%), caryophyllene oxide (2.29%), 4-terpineol (1.42%), Calamenene (1.33%), 1,8-cineol (1.116%) were determined.

Table 1. The percentage composition of *N. italica* subsp. *cadmea* oil detected by GC-MS and GC-FID analysis.

Compounds	(%)
α-Pinene	0,52
Sabinene	0,44
α-Terpinene	0,26
1,8-Cineole	1,16
γ-Terpinene	0,51
Linalool	3,97
Caryophyllene	6,89
4-Terpineol	1,42
α-Copaene	0,35
α-Humulene	0,33
Lavandulol	0,93
α-Terpineol	0,35
Germacrene	0,86
γ-Cadinene	0,52
Calamenene	1,33
Caryophyllene oxide	2,29
Nepetalactone (Total isomers)	74
Other Compounds	3,87
Total	100

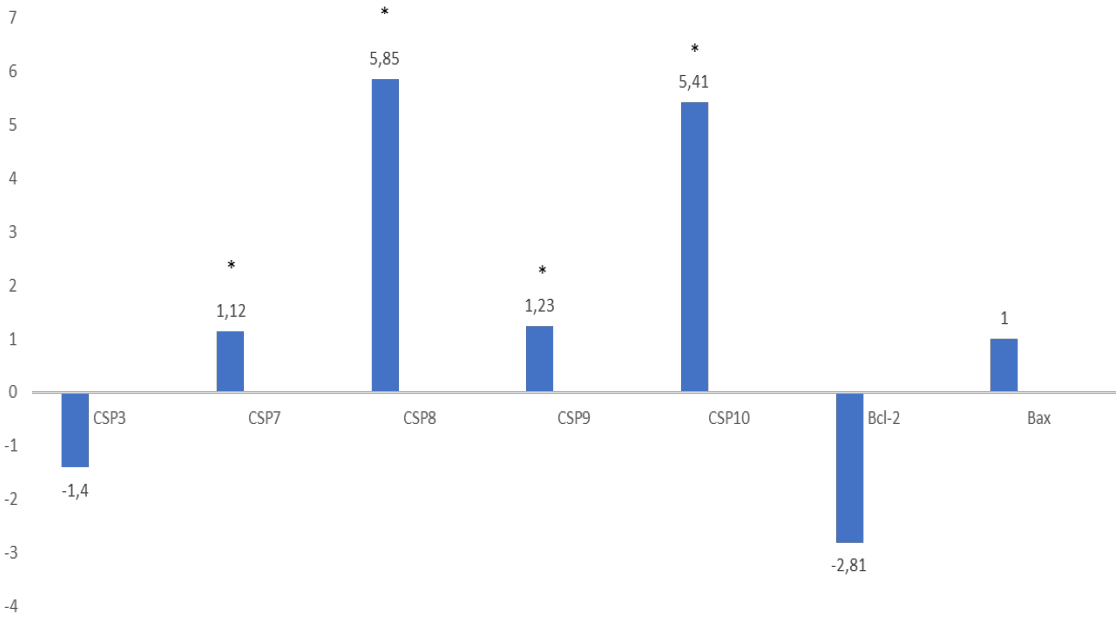
Figure 1. The chromatogram of *N. italica* subsp. *cadmea* oil detected by GC-MS and GC-FID analysis.



3.2. Apoptotic Effects of *Nepeta*

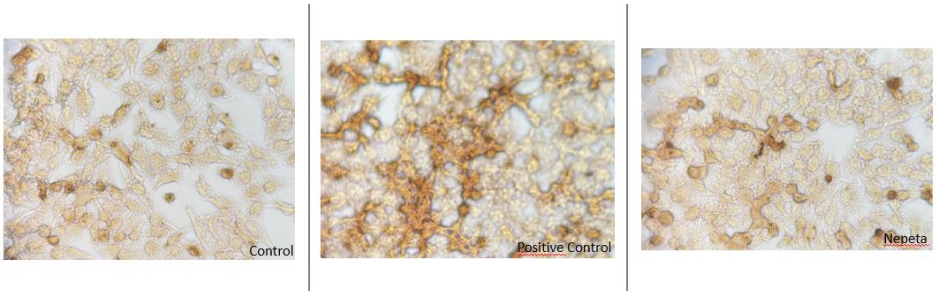
To investigate whether the growth inhibitory effect of *N. italica* subsp. *cadmea* oil is associated with apoptosis, expressive quantity of apoptosis-related genes such as Bcl-2, Caspase 9, Bax, Caspase 3, Caspase 8, Caspase 7, Caspase 10 was carried out. Caspase8, Caspase 10 were overexpressed while Bcl-2 was downregulated (Figure 2).

Figure 2. Fold Regulation of Apoptosis Related Genes. * indicates statistically important (p<0.05)



Tunel assay results showed apoptotic cells in control and induced group (Figure 3). The cells with brown staining were TUNEL-positive cells, namely the apoptotic cells. We found similar positive cells in the Nepeta-treated group like in the control group.

Figure 3. Apoptotic levels of *Nepeta* induced cells.



Essential oils of *Nepeta* species such as *N. ucrainica* L. spp. *kopetdaghensis, N. cataria* var. *citriodora* (Becker)*, N. sintenisii Bornm, N. cataria L.,* and *N. menthoides Boiss & Buhse* have been declared to have cytotoxic activity in various human cancer cell lines (Sharma *et al*., 2021).

Apoptosis stimulated by *N. cataria L.* in human prostate and breast cancer cells under in vitro conditions. These extracts showed much more cytotoxic effects against estrogen-receptor-positive prostatic cell line PC3 compared to low hormone receptor expressing prostatic cells DU-145 (Emami *et al*., 2016). *E*ssential oils of *N. cataria L.* and *N. cataria* var. *citriodora* have been demonstrated to exhibit cytotoxic effect against both BEAS-2B human bronchial epithelial and HaCaT human keratinocyte cell lines (Suschke *et al*., 2007).

N. deflersiana ethanolic extracts' (NDEE) cardioprotective effect against isoproterenol (ISP)-induced myocardial damage in rats was investigated. It is suggested that NDEE treatment can obstruct oxidative stress, inflammation, and programmed cell death, thus making an effective approach against myocardial apoptosis. It has been showed that NDEE has potent cardioprotective, antioxidant, anti-apoptotic and anti-inflammatory potential opposed to myocardial damage (Al-Taweel *et al*., 2017).

It has been reported that *N. menthoides* has an anti-apoptotic effect on axotomized spinal motoneurons. *N. menthoides* can induce a neuroprotective effect on axotomized dorsal root ganglion sensory neurons by reducing apoptotic cell death (Delshad and Parvizi, 2014). Likewise, the anti-tumoral properties of *N. menthoides Boiss & Buhse* were studied. The cytotoxic activity of the essential oil opposed to T47D breast ductal carcinoma, HT-29 and Caco-2 colorectal adenocarcinoma, and NIH-3T3 fibroblast cells has been described (Kahkeshani *et al*., 2014).

The presence of the main volatile component, which is nepetalactone derivatives, is associated with the cytotoxic potential of the essential oil. Coated with nepetalactone extracted from *N. gloeocephala* Rech f. treatment induced apoptosis in Hela cervical carcinomas. Phosphorylation of integrin-related kinase in Thr-173 and Ser-246 was decreased by SiO_2-antisense molecules without affecting integrin-related kinase levels and gave rise to cell cycle arrest in G2/M. In addition, SiO2-antisense coated with nepetalactone decreased downstream AKT-Thr308 phosphorylation and AKT-Ser473 devoid of affecting the total amount of protein kinase B/AKT (Ashkezary *et al*., 2017).

N. ucrainica L. spp. *kopetdaghensis* essential oil exhibit cytotoxic effects against both ovarian carcinoma A2780 and mammary adenocarcinoma MCF-7 cell lines (Shakeri *et al*., 2016). *N. curvidens* essential oil's cytotoxic, antifungal, and antimicrobial activities were investigated in vitro. Also, anti-inflammatory and antinociceptive effects were appraised in vivo. The cytotoxicity against human lung cancer A549, human oral epidermal carcinoma KB, bladder epithelial carcinoma C450 was appraised. Meanwhile, A549 lung cancer cell line has

the highest inhibition at the lowest concentration of the essential oil. The C450 bladder cancer cell line, was the most resistant to the essential oil. Furthermore, the essential oil showed mild toxicity for the human normal line and high toxicity for cancer cell proliferation (Ashrafi *et al*., 2020).

N. govaniana essential oil displayed depending on the concentration growth inhibition with cell line linked growth of cancer cells and has highest activity towards A-549 lung cancer cell line, followed by Colo-205 colon cancer cell line (Dar *et al*., 2014).

The cytotoxic potential of the extract *N. glomerata* Montbret & Aucher ex Bentham plant has been shown to be more active in the renal adenocarcinoma cell line (48%) then amelanotic melanoma (28%) (Rigano *et al*., 2011). The cytotoxic and cell viability effects of niosomal form of the *N. persica* Boiss. extract in MG63 bone cancer cells were evaluated. It has been stated that *N. persica* extract has anti-cancer effects (Aboeepoor *et al*., 2020).

The cytotoxicity of *N. juncea* Benth. extracts were studied in MCF-7 human breast adenocarcinoma cells and human HepG2 human hepatocellular carcinoma cells and *N. juncea* extracts showed antiproliferative activity in MCF-7 and HepG2 cells at a dose-dependent manner. It was also reported that of *N. juncea* methanolic extracts exhibit maximum cytotoxic effect in both cancer cell lines, while minimal cytotoxic effect was observed in aqueous extracts (Sharifi-Rad *et al*., 2020).

Anticancer and antimicrobial effects of active phytochemicals of ethanol extract (NDE) obtained from the aerial parts of *N. deflersiana* (ND) and NDE identified by gas chromatography-mass spectroscopy (GC-MS) were investigated. NDE's lung cancer NCI-H460 inhibited the proliferation of A549 cells. NDE demonstrated a dose-dependent inhibition in cancer cell migration. A dose-sensitive increase in early and late phase apoptosis was investigated in these cancer cells. While significant increases were noted in Bax and Caspase 3 levels, NDE decreased anti-apoptotic Bcl-2 levels in both cell lines. Ahmad et el. explained that NDE has anticancer effects on lung cancer cells, which may involve mitochondria-mediated apoptosis (Ahmad *et al*., 2020).

The cytotoxic effects of monoterpenes have previously been showed against different cancer cells. 1,8-cineol, the major compound found in *N. binaloudensis* oil, has been demonstrated to inhibit the proliferation of HepG2 and A549 cells (Kladniew *et al*., 2014). In another study showed that 1,8-cineol supressed proliferation of colon cancer cells by activating apoptosis (Murata *et al*., 2013). Alpha terpineol has been declared to inhibit the growth of cancer cells through a mechanism that includes inhibition of the NF-kB pathway (Hassan *et al*., 2010). At a concentration of 320 µg/mL, γ-terpineol induced typical genome DNA fragmentation after 12, 24, 36, 48 hours and hallmark apoptosis in multiples of 180-200 bp. γ-terpineol not only induced apoptosis in human liver cancer BEL-7402 cell lines, but also greatly reduced BEL-7402 cell protein expression (Wu *et al*., 2014). The anti-tumor effect of α-pinenin has been reported on human hepatoma cell lines by inducing G2/M cell cycle arrest (Chen *et al*., 2015). α-pinenin has been shown to inhibit BEL-7402 cells by arresting cell growth in the G2/M check point via down-regulating Cdc25C expression and decreasing cycle dependence on kinase 1 (CDK1) (Chen *et al*., 2014).

4. CONCLUSION

This study was carried out to confirm the cytotoxic and apoptotic effect of essential oil obtained by hydrodistillation from the aerial parts (flower, leaf, seed and stem) of *N. italica* subsp. *cadmea.* Various studies have been made to appraise the anti-apoptotic, anticancer, and cytotoxic potentials of different essential oil extracts from different species of the genus *Nepeta*. Extracts of these essential oils can be exerted by a variety of mechanisms, including cytotoxic and anti-cancer potential, increased membrane permeability, depolarization of cell membrane

integrity and disruption, decreased activity of membrane-bound enzymes, modification of the mevalonate metabolism pathway, and apoptosis induction. This study will contribute to the literature about effects of *N. italica* subsp. *cadmea* and its anticancer activities in Saos-2 osteosarcoma cells.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Siddika Boztas: Material preparation, data collection, analysis and edited. **Buket Er Urganci:** Material preparation, data collection, analysis and first draft of the manuscript. **Mucahit Secme:** Material preparation, data collection, analysis and edited. **Nazan Keskin:** Read and approved the final manuscript.

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Embryogenic Callus Differentiation in Short-Term Callus Derived from Leaf Explants of Alfalfa Cultivars

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Abstract

Alfalfa is a forage crop that accounts for one of the best sources of protein and is commonly grown all over the world. *In vitro* callus and embryogenic induction of alfalfa have been investigated previously; however, most of the investigations were almost limited with callus formation. In this study, leaves of 5 *Medicago sativa* L. cultivars (Alsancak, Sazova, Plato Iside, and Bilensoy) have been used. Influences of culture medium contents and hormones applications on callus and embryogenic callus induction were determined to optimize *in vitro* culture mediums of alfalfa (0.0125 g kinetin 2,4 D 1 mg mL^{-1}; 1 g kinetin 2,4 D 1 mg mL^{-1} ; 0.25 g kinetin 2,4 D 2 mg mL^{-1}; 0.5 g kinetin 2,4 D 2 mg mL^{-1}). Callus formation was detected at a rate of 74% in 5 different cultivars used in the experiment. The five *alfalfa* cultivars were classified into four categories in terms of embryogenic differentiation capacity. The tested alfalfa cultivars varied in their callus formation and embryogenic callus differentiation. Sazova, Alsancak, and Bilensoy were detected for better callus formation; similarly, the same cultivars responded with better embryogenic callus formation in the culture mediums including various hormone concentrations. The present study shows that our methods have beneficial impacts on the somatic embryo induced by alfalfa. However, it depends strongly on genotype, hormone concentrations and the other medium components.

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1. INTRODUCTION

Alfalfa is one of the significant forage plants which is commonly used in animal feed due to its high protein, carbohydrate, and vitamins in many regions. It is a well-known forage queen and crops domesticated in ancient times by humans (Nofouzi *et al*., 2019; Yazıcılar *et al*., 2021). Turkey has been considered the secondary center of genes for alfalfa crops. Alfalfa is grown for its animal feed nutrition and stress adaptation, large disease resistance, and high quality, as well as its function in soil protection, and bioremediation, nitrogen cycle, wildlife habitat (Heuze *et al*., 2012). Alfalfa has emerged as heterozygote, cross pollination, mixploid and perennial. Therefore, *in vitro* culture applications are very difficult. *In vitro* cell and tissue culture strategies are quiet impact techniques that use morphological and physiological-based unique methods to promote the yield of crop and plant products, or to minimize the stress that causes biotic and abiotic environmental factors, thus blocking crop yield losses (Bezirganoglu, 2017). Several reports indicated on agronomic traits such as vegetative biomass, row protein and cellulase obtained by traditional breeding methods, and a repeated regeneration system should be done from in vitro cultures to benefit effectively from biotechnological treatments used in cultivar breeding (Zhang *et al*., 2010). Callus is a primary tool in order to induce embryogenic callus and somatic embryos in crops. Callus of alfalfa or any explants is

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essentially a more and fewer undifferentiation structure which generally develops on wounds of exogenous stimulates (Dos Santos et al., 1983; Fehér, 2015; Sangra *et al*., 2019). Somatic embryo induction is linked to the ability of embryogenic callus formation. However, plantlets developments are not able in all somatic embryos on the embryogenic callus. Samac and Austin-Phillips (2006) reported that tissue culture formation of callus in alfalfa crop is usually initiated with the supplementation of 2,4-D (2,4-Dichlorophenoxyacetic acid) and kinetin hormones to the embryogenic maintenance medium. Despite all efforts, most forage crops and alfalfa especially have evident to be very tedious for the somatic embryo of embryogenic calli. The ability of somatic embryos to be converted into plantlets depends on several factors such as embryo maturation, synthesis and accumulation of storage compounds, and development of desiccation tolerance. Alfalfa callus was earlier obtained by several researchers from hypocotyl tissue (Brown *et al*., 1988), the apical meristem and petiole organs (Fujii *et al*., 1990), and leaf (Nikolic *et al*., 2010). The current report was performed to define the optimization of various alfalfa cultivars into tissue culture of different plant growth regulators and doses.

2. MATERIAL and METHODS

2.1. Plant Material and Culture Media

Dimethyl sulfoxide (DMSO) (Merck) was used as a negative control and dissolving compound. For antimicrobial analyzes, Luria Bertaini Agar (LBA) (Difco) Mueller Hinton Agar (MHA) (Oxoid) and Mueller Hinton Broth (MHB) (Biolife), Netilmicin disc (Bioanalyse) were used as positive controls.

2.2. Embryogenic Callus Induction

Three months after callus formation, embryogenic differentiation from the healthy callus formed on the explants was initiated on MS basal medium with 0.0125 g kinetin 2,4 D 1 mg mL^{-1}; 1 gr kinetin 2,4 D 1 mg mL^{-1}; 0.25 g kinetin 2,4 D 2 mg mL^{-1}; 0.5 g kinetin 2,4 D 2 mg mL^{-1}. Embryogenic differentiation appeared at 28 ^{o}C under a photosynthetic photon flux density of 7 mmol m^{-2} s^{-1} within 3 months The intact callus was sub-cultured at 3-week periods. Callus was weighted on callus formation medium.The number of callus differentiation per callus (0.5 cm in diameter, average fresh weight 60 mg) was scored on embryogenic callus formation medium.

2.3. Statistical Analysis

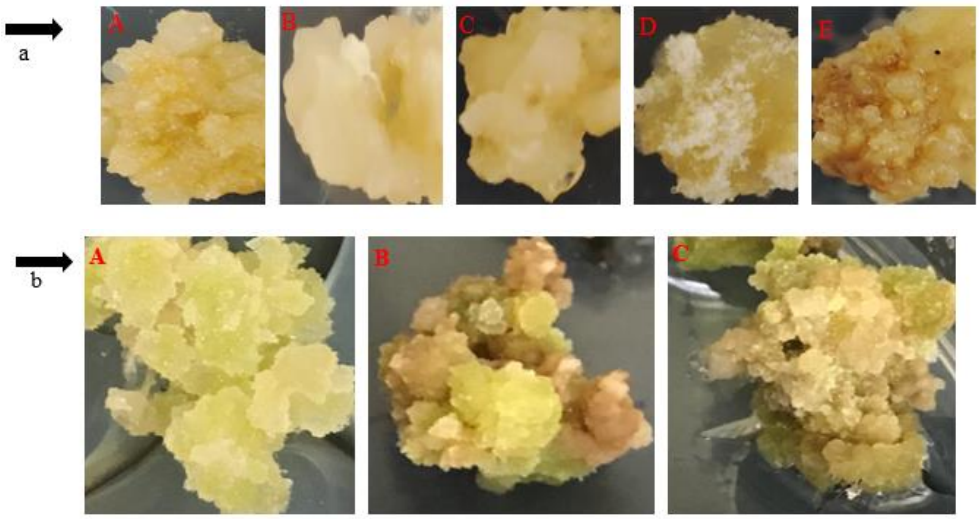
The statistical analysis was carried out in factorial design using a Completely Randomized Design (CRD) with 3 replications of 25 explants per Petri dish. Each plate is evaluated as one experimental unit. Analysis of variance and the Duncan test were used to determine significant differences. SPSS (25.0) statistical program was used for all computations.

3. RESULTS and DISCUSSION

Plant regeneration through *in vitro* culture in many crops depends on efficient somatic embryos through callus formation. Although embryos are the common initial material of explants for plant regeneration via somatic embryogenesis, the use of leaf explants has considerable superior compared to embryos as explants in forage crops (Kumar *et al*., 2012; Liu et al., 2013). However, success in tissue culture studies in many plant species including forage crops fairly depends on genotype, explant sources and nutrition components. In particular alfalfa *in vitro* embryogenic callus formation is strongly influenced by the genotype and its influence on somatic embryo is far greater than the medium composition. Therefore, the optimization of *in vitro* conditions can be beneficial for the genetic enhancement of alfalfa. The culture medium optimization can be affected by many traits, such as explant sources, genotype,

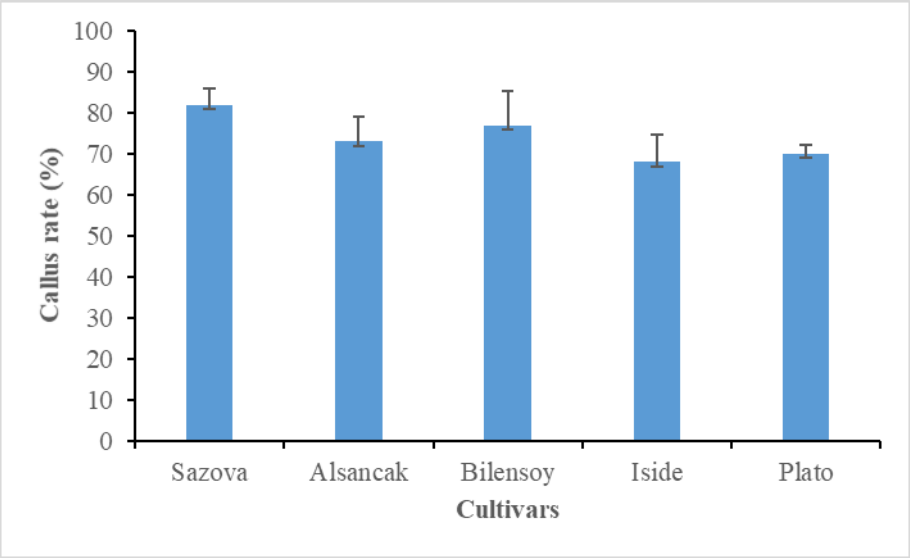
growth conditions and medium composition (Yazıcılar et al., 2021). The combination of cytokinins and auxins promoted callus induction in a number of plant species (Choudhry et al., 2014). 5 cultivars that must be tested capable of producing leaf explants. The ability to form embryogenic differentiation and somatic embryoids varied remarkably. The cultivars with tissue culture embryogenic capacity are displayed in Figure 1. Two cultivars including Plato and Iside produced callus tissues and not embryogenic differentiation.

Figure 1. (a) Changes in callus maintenancein alfalfa genotypes combination kinetin-2.4 -D (A: Aazova, B: Alsancak, C: Bilensoy, D: Iside, E: Plato), (b) Changes in embryogenic callus maintenance in alfalfa genotypes combination kinetin-2.4 -D (A: Sazova, B: Bilensoy, C: Alsancak).



However; they produced effectively callus formation in tested media conditions. The remaining cultivars were able to capacity produce embryogenic differentiation. The response of embryogenic differentiation was dependent on genotype and media conditions. Various types and concentrations of 2,4-D and kinetin affected callus formation ratio% at 30 days of experiments. Callus formation was detected after 30 days of leaf culture explants in a culture medium including 2,4-D and various kinetin concentrations. Callus formation was detected at a rate of 74% in 5 different cultivars used in the experiment. After callus formation, the healthy callus was transferred to light conditions for embryogenic differentiation. Embryogenic callus with green plantlets were considered as embryogenic callus with somatic embryos potential. The difference between genotypes in terms of embryogenic callus formation rate according to callus number was found to be statistically significant ($p<0.05$). Kumar *et al*. (2012) confirmed the impacts of TDZ (Thidiazuron) on *in vitro* direct regeneration of shoot apical meristems supported by some parts of hypocotyl and leaf explants. They were exposed to 1 mg L^{-1} BAP (6-Benzylaminopurine), and 2 mg L^{-1} TDZ to direct organogenesis. They detected a regeneration frequency of 67-93% and five Indian alfalfa genotypes responded positively to the *in vitro* regeneration procedure. Our findings displayed that callus induction rates from 82% to 68%, recommending great genotypic variations in the callus development capacity among the five cultivars (Figure2 and Table 1).

Figure 2. The callus formation ratio of callus tissues from 5 alfalfa cultivars.



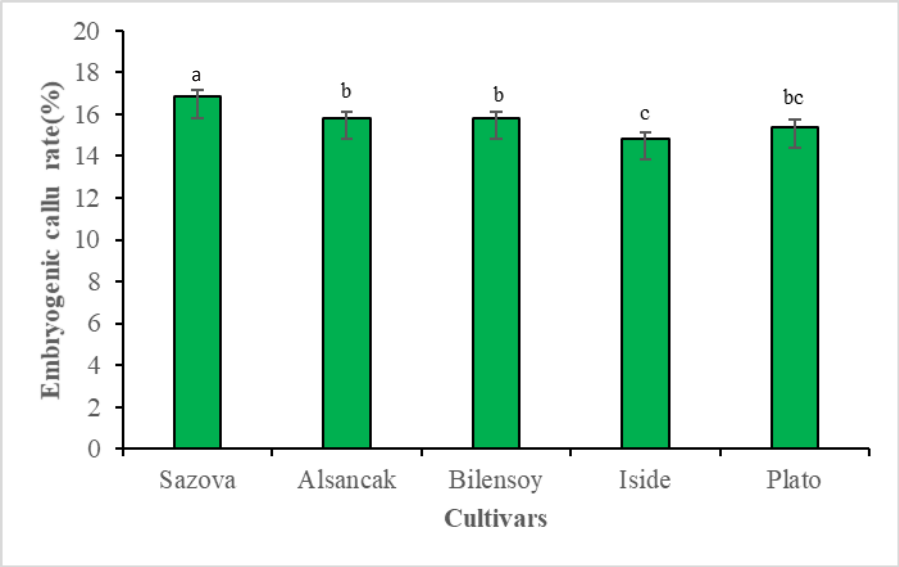
[a,b] Means in the same column with various superscript letters differ significantly ($p \leq 0.05$)

Table 1. Changes in embryogenic callus maintenance in alfalfa genotypes combination auxin-melatonin.

Cultivars	Callus Rate (%)	Embryogenic Callus Rate (%)
Sazova	82±4,000[a]	16,83±0,318[a]
Alsancak	73±6,000[ab]	15,83±0,318[b]
Bilensoy	77±8,240[a]	15,83±0,318[b]
Iside	68±6,530[b]	14,83±0,318[c]
Plato	70±2,300[b]	15,41±0,318[bc]

[a,b] Means in the same column with various superscript letters differ significantly ($p \leq 0.05$)

Figure 3. The embryogenic callus formation ratio of callus tissues from 5 alfalfa cultivars.



[a,b] Means in the same column with various superscript letters differ significantly ($p \leq 0.05$)

The 5 alfalfa cultivars (Sazova, Alsancak, Bilensoy, Iside, and Plato) had been classified into four categorised in terms of embryogenic differentiation capacity (Figure 3). The cultivar Sazova was 16.83%, in 2,4-D + 1mg L^{-1} kinetin as the first category, Alsancak and Bilensoy became 15,83% as the second category, followed by Plato 15,41% as in the third category, and Iside 14,83 as fourth categories. This report displayed clearly that influenced the embryogenic callus differentiation parameters of alfalfa leaf explants tissue culture. A Similar result was detected by Varga and Badea (1992). They investigated the effect of *in vitro* plant regeneration ability and a series of three culture mediums by using alfalfa leaves culture. They reported that responses of the leaf alfalfa callus tissues to the embryogenic induction were different depending on the type of auxin;cytokinin balance, their concentration sucrose amount, and genotype. The findings confirm the impact of genotype and hormone dosages on callus initiation, embryogenic differentiation, regeneration frequency, and the number of regenerated shoots.

4. CONCLUSION

We successfully detected the embryogenic callus formation in five alfalfa cultivars and displayed the significant differences among alfalfa cultivars of the embryogenic callus development potential. The tested alfalfa cultivars exhibited an improvement in survival rate in response to *in vitro* callus and embryogenic callus formation. The embryogenic callus developed in the present study seems to be useful in increasing the somatic embryos and plant regeneration from embryogenic callus.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Busra Yazicilar: Writing, editing, validation. **Yen Ling Chang:** Experiment design, supervision, laboratory work, statistical analysis.

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Biological Evaluation of Azide Derivative as Antibacterial and Anticancer Agents

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Acinetobacter baumannii,
Candida albicans,
Candida parapsilosis

Abstract

Azides are significant compounds because of their biological effects on bacteria, viruses, fungi, and cancer. The antibacterial and anticancer properties of azide derivatives have been demonstrated in several microbial strains and cancer cell lines. The most significant microorganisms in command of hospital-acquired nosocomial infections include *Acinetobacter baumannii* (*A. baumannii*), Methicillin-resistant *Staphylococcus aureus* (MRSA), *Candida albicans* (*C. albicans*), and *Candida dubliniensis* (*C. dubliniensis*). These infections have become resistant to a variety of antibiotics. These resistant infections are frequently seen in cancer patients. Novel drug-active compounds are thus required that have both antibacterial and anticancer activity. This study examined previously synthesized *1-azido-5,6,7-trimethoxy-2,3-dihydro-1H-indene* **(4)** for its antibacterial and anticancer activities. Antimicrobial activity was assessed using the disc diffusion technique, and minimum inhibitory concentration values were computed for zone formation in the examined pathogens. The study's findings indicate that while azide derivatives did not exhibit any effect against other pathogens, they suppressed bacterial growth with a zone diameter of 10 mm against *A. baumannii*. The MIC values of azide derivative against *A. baumannii* were 3.90 µg/ml. WST-8 analysis in Caco-2 cancer and healthy fibroblast cell line was used to determine the anticancer study. As a result of this analysis, the IC_{50} value was calculated to be 2.99 µM. There is little anticancer activity. A very low toxic effect of the azide compound on fibroblast cells was also observed. These findings suggest that this azide derivative may be tested as a potential antibacterial and anticancer agent.

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1. INTRODUCTION

Azides are organic compounds with a functional group (N_3) that are very energetic in chemical reactions. Because azides are the precursor amines, it also takes part in the "click and Staudinger reactions" (Karimov *et al*., 2016; Tishchenko and Myznikov, 2022). Azide compounds have antiviral (Araújo *et al*., 2020), anticancer (Nicolaus *et al*., 2010), and antimicrobial properties (Phetsang *et al*., 2014). Commercially, a derivative of zidovudine **1** azide is used to treat HIV infections (Cooper and Lovett, 2011). Azide derivatives are also employed as precursor compounds in the synthesis of some drugs that are used commercially. It has been shown that aryl azide derivatives are effective anticancer drugs. For instance, a *trans*-2,3-dimethoxycinnamoyl azide **2** derivative improved the anticancer effects of romidepsin on bladder cancer cells in vitro and in vivo. The aryl azide counterpart of

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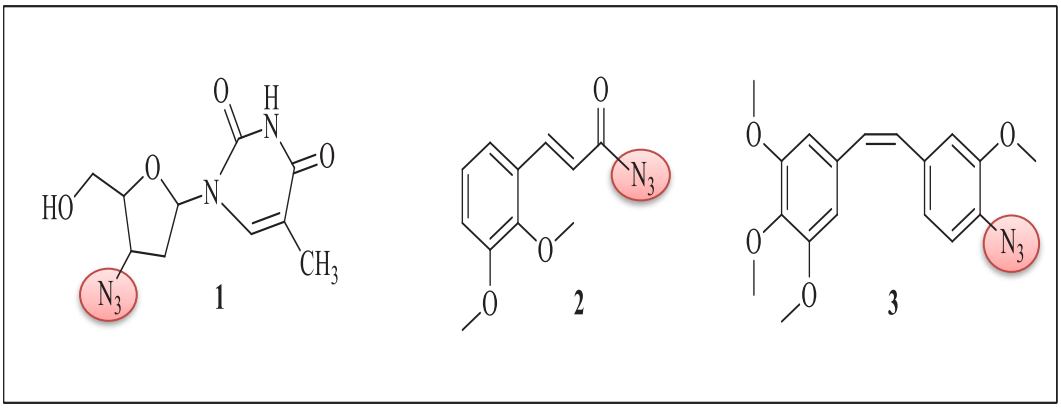
combretastatin A-4 **3** demonstrated strong anti-tubulin activity (Figure 1) (Fu *et al*., 2017). Azide derivatives transform compounds into drug forms *in vivo* because the compounds in their azide form have longer half-lives and more effectively cross the blood-brain barrier (Kotra *et al*., 1996).

The Gram-negative bacillus bacterium *A. baumannii* is one of the most challenging resistant organisms to eradicate (Wei *et al*., 2021). The most well-known feature of the species, *A. baumannii*, is its capacity to infect immunocompromised humans. It has genes that render it immune to antibiotics capacity to cure *A. baumannii*, making it a prevalent resistant bacterium in hospitals (Kyriakidis *et al*., 2021).

Figure 1. Some biologically active azide derivatives **1-3**.



Infections acquired in hospitals are, therefore, far more common and pose a major risk. Meningitis, skin and soft tissue infections, wound infections, urinary tract infections, and other uncommon nosocomial diseases have all been linked to *A. baumannii* (Pompilio *et al*., 2021). A significant death rate is also associated with pneumonia infections due to the bloodstream and ventilator usage (Gedefie *et al*., 2021).

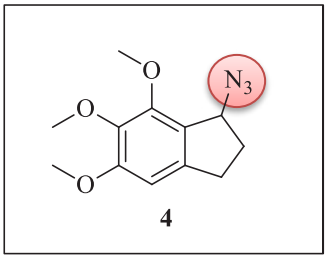
Gram-positive MRSA is genetically distinct from other strains of *Staphylococcus aureus*. People who have MRSA infections struggle to recover from them. MRSA is a disease that is frequently seen in both hospitals and the general population. MRSA is a common cause of bacteremia, septicemia, endocarditis, skin and soft tissue infections, and bone infections as a hospital-acquired infection (Abdullahi *et al*., 2021). Penicillin was the first antibiotic used to treat severe *S. aureus* infections, but once it was utilized, resistance soon emerged. Methicillin was created because of research into novel antibiotics to treat penicillin-resistant *S. aureus* bacteria. Unfortunately, MRSA first appeared in the UK in 1960 (Kriegeskorte *et al*., 2012).

A significant source of morbidity and mortality in hospitals is invasive candidiasis. *C. albicans* is prevalent in human microflora organisms, even though opportunistic human infections are the primary cause of candidemia and candidiasis globally (Herrera-Hidalgo et al., 2020). A switch in morphologies is a key component of pathogenicity. Even though *C. albicans* is the most common cause of invasive *candidiasis* and infections brought on by non-*C. albicans* species are on the rise (Lopes and Lionakis, 2022). An opportunistic human pathogen called *C. dubliniensis* is increasingly being blamed for nosocomial bloodstream infections in hospitalized patients. The intravascular devices that cause candidemia have also been related to *C. dubliniensis* (Sullivan and Coleman, 1998).

Colorectal cancer is the third most common leading cancer in men and women, resulting in many deaths of this type of cancer (Kabra *et al*., 2009). Despite aggressive treatments, many tumors are a type of cancer highly resistant to current treatments (Lea, 2015).

In this study, the antimicrobial activities of the previously synthesized 1-azido-5,6,7-trimethoxy-2,3-dihydro-1H-inden (Figure 2) (Ozgeris *et al*., 2015) were evaluated against *A. baumannii*, MRSA, *C. albicans,* and *C. dubliniensis*. Its anticancer activity was investigated in the Caco-2 cancer cell line, one of the most widespread cancer types, and its cytotoxic property was also investigated in the healthy fibroblast cell line.

Figure 2. Azide Derivative.



2. MATERIAL and METHODS

2.1. Chemicals and Materials

Dimethyl sulfoxide (DMSO) (Merck) was used as a negative control and dissolving compound. For antimicrobial studies, Mueller Hinton Agar (MHA) (Oxoid) and Mueller Hinton Broth (MHB) (Biolife), Netilmicin disc (Bioanalyse), and amphotericin B (Sigma Aldrich) were used as positive controls. Dulbecco's Modified Eagle Medium (DMEM) (Biowest), Fetal Bovine Serum (FBS) (Gibco), L-Glutamine (WISENT INC), PenStrep (Gibco), Phosphate Buffer Saline (PBS) (Gibco), Hydrogen peroxide (Sigma Aldrich), Trypsin/EDTA (Gibco), CVDK-8 (Ecotech) was procured and used.

2.2. Strains of Bacteria and Fungi

The research included an examination of the compound **4** antibacterial properties. *A. baumannii* ATCC BAA-1605, MRSA ATCC 43300, *C. albicans* ATCC 10231, *and C. dubliniensis* CBS 7987 were the strains used in this study. Antimicrobial activity was evaluated using the minimum inhibitory concentration (MIC) and disc diffusion techniques.

2.2.1. Research on Microbes Utilizing Disc Diffusion Analysis

For the disc diffusion test, the microbial inoculum was developed in line with 0.5 McFarland and then spread on Petri dishes. Compound **4** synthesized at 10 µg/ml in three replications (5 µg active compound + 250 µl DMSO + 4750 µl dH_2O) was adsorbed onto discs and placed in Petri dishes. The antibacterial susceptibility of isolates was tested using the zone diameters obtained after 24 hours of incubation at 37°C for bacterial strains and 30°C for fungal strains. Positive controls for the study's antibacterial and antifungal tests were Netilmicin and amphotericin B, respectively. In each test, DMSO was utilized as a negative control (Ozgeris, 2021).

2.2.2. MIC-Based Antimicrobial Research

A disc diffusion experiment was used to assess the activity of compound **4**, and the MIC values were calculated. To calculate the MIC values, 100 µl of MHB and 100 µl of the synthetic medication were added to each well of the 96-well plates. Using the serial dilution method, 100 µl of 0.5 McFarland bacterial culture inoculum was introduced to the wells to change the

concentration. The turbidity in the wells was measured spectrophotometrically (at 600 nm) after the 24-hour incubation period, and MIC values were calculated (Özgeriş *et al*., 2021).

2.2.3. Cell Culture Method, Toxicity Research, and the WST-8 Test

To maintain pH balance, Caco-2 and Fibroblast cells were grown in a DMEM medium with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine at 37°C in an incubator. Cells were passaged for several days, depending on the experiment's aims and cell density. WST-8 is a monosodium salt (Tetrazolium salts*)* of the cytotoxicity and metabolic activity assay compound. This non-radioactive spectrophotometric assay uses tetrazolium salts to discriminate between live cells. The "Cell Viability Detection Kit-8 (CVDK-8)" kit was employed in the investigation to assess the dose-dependent cytotoxic and anti-proliferative effects of compound **4**. study used the "Cell Viability Detection Kit-8 (CVDK-8)" kit to evaluate the dose-dependent cytotoxic and anti-proliferative effects of the compound **4** synthesized on healthy and cancer cell lines in accordance with the procedure. This was accomplished by seeding a 96-well plate with $7x10^3$ cells in each well and incubating it for 24 hours. After that, the substance was added and incubated for 48 hours at the given dose concentrations (200, 100, 50, 25, 12.5, 6.25 µM). At the end of the period, 10% of the well volume WST-8 solution was applied to each well in a clean, dark environment. After the produced cultures were incubated for 4 hours at 37°C in a 5 % CO_2 incubatory, the absorbance of the created formazan crystals was measured in a spectrophotometer instrument at 450 nm, and the percent viability and cytotoxicity values were computed for each dosage given. As a positive control, the cells in the H_2O_2-treated wells worked better than those in the untreated wells (Kaci *et al*., 2022).

2.3. Statistical Analysis

The study's data was subjected to statistical analysis using the GraphPad Prism 9.3.0 software suite.

3. RESULTS and DISCUSSION

3.1. Chemicals and Materials

3.1.1. Antibacterial Effect

Using disc diffusion and microdilution techniques, the antibacterial activity of compound **4** against MRSA and *A. baumannii* strains was assessed (Table 1). The study's findings demonstrated that the azide compound has antibacterial activity against *A. baumannii* isolates but not against MRSA isolates.

According to the study, compound **4** minimum inhibitory concentration (MIC), which exhibits antibacterial activity in *A. baumannii*, is 3.90625 µg/ml.

Table 1. Disc diffusion analysis results of azide compound

Bacteria Strain	(4)	Control**
A. baumannii ATCC BAA-1605	1.0*	1.2
MRSA ATCC 43300	No zone	1.0

* zone diameter (cm) ** Positive control: netilmicin

3.1.2. Antifungal Properties

Disc diffusion was used to assess the antifungal activity of compound **4** against isolates of *C. albicans* and *C. dubliniensis*. According to the study, compound **4** demonstrated no antifungal effects on isolates of *C. albicans* and *C. dubliniensis*. (Table 2).

Table 2. Disc diffusion analysis results of azide compound

Fungal Strain	(4)	Control**
C. albicans ATCC *10231*	No zone	1.0
C. dubliniensis CBS 7987	No zone	1.1

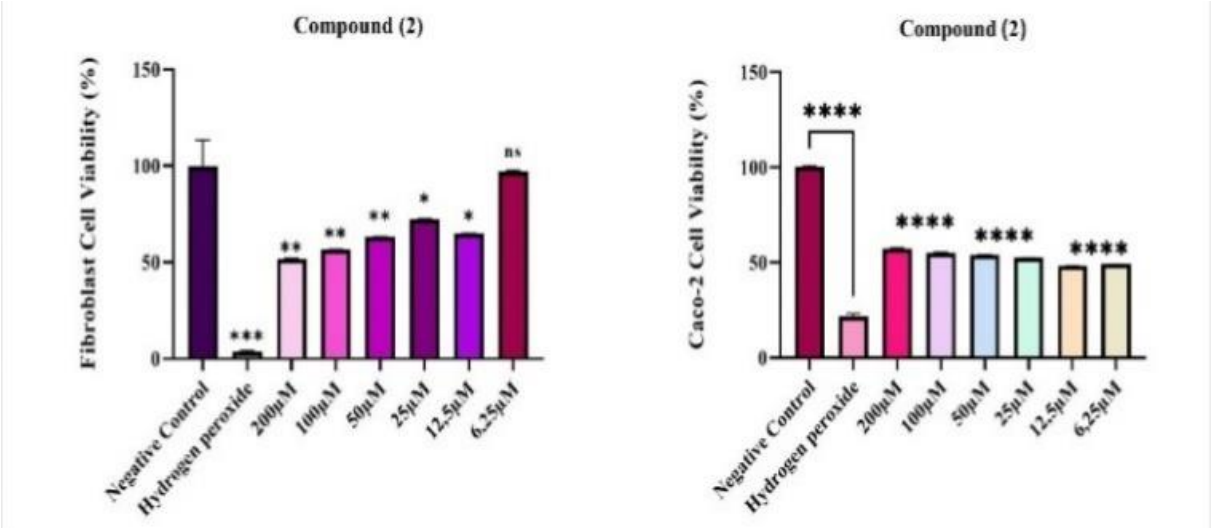
*zone diameter (cm) ** Positive control: Amphotericin B.

It is well-known in the literature that azide compounds have antimicrobial activity (El Rayes, 2010; Kalelkar *et al*., 2019; Sharma *et al*., 2014). In some studies, antifungal activity was observed in addition to the antibacterial activity of azide compounds (Obafemi and Akinpelu, 2005). It has even been reported to have good activity on Candida-type yeasts (González-Calderón *et al*., 2016). However, it was observed that compound **4** has antibacterial activity but not antifungal activity in our study. This result is thought to be because structures with the same functional group do not show the same biological activity. The activities of azide derivatives against Gram-negative and Gram-positive bacteria were compared in the studies. According to the study, Gram-negative bacteria respond better to azide derivatives than Gram-positive bacteria because of their distinct cell walls (Sarwar *et al*., 2015). Compound **4** showed no effect on MRSA, a Gram-positive bacterium, in our antibacterial activity testing. Still, it did inhibit the growth of *A. baumannii*, a Gram-negative bacterium, and its MIC value was low. The study is in line with the body of research.

3.1.3. The Effects of Compound 4 on Cell Viability and IC_{50} Value were Examined Using the WST-8 Assay.

The WST-8 test was employed to examine the compound's impact on the Caco-2 and fibroblast cell lines utilized in the investigation. The vitality of cells exposed to different compound concentrations for 48 hours (6.25 µM, 12.5 µM, 25 µM, 50 µM, 100 µM, and 200 µM) was assessed separately and compared to the control. (Figure 1). The calculations showed that the azide derivative statistically increased the viability of Fibroblast cells, while Caco-2 decreased cell viability. Caco-2 cell viability (%) was used to calculate the IC_{50} value. In compound **4** Caco cells, the IC_{50} value was 2.99 µM.

Figure 1. WST-8 analysis results of azide compound



It is well-known that azide compounds have anticancer activity (Nicolaus *et al*., 2010; Tanabe *et al*., 2012; Thamilarasan *et al*., 2015). Azide derivatives have been proven in studies to have anticancer activity. The IC_{50} was determined as 5.25 µM, and azide derivatives were shown to have anti-proliferative effects in numerous cancer lines (Fu *et al*., 2017). According to research, azide derivatives have demonstrated anticancer activity in colon cancer cell lines and exhibited low toxicity in healthy fibroblast cells, where the IC_{50} value was estimated as 6.27 µM in the colon cancer cell line (Sarwar *et al*., 2015; El-Kardocy *et al*., 2019). In our work, compound **4** showed an anti-proliferative effect on Caco-2 cells, and the IC_{50} was estimated to be 2.99 µM however, healthy fibroblast cells did not show any toxic effects. Compound **4** employed in our investigation is consistent with the literature considering these findings, and, interestingly, the IC_{50} value is low compared to other studies.

4. CONCLUSION

Consequently, antibacterial, anticancer, and toxic effects of the compound **4** were examined. Compound **4** of MRSA, *C. albicans*, and *C. dubliniensis* showed little action. Compound **4** showed only antibacterial activity against the isolate of *A. baumannii*. Furthermore, while it had anticancer action in the Caco-2 cancer cell line, it did not demonstrate any toxic effect in the healthy fibroblast cell line. Azide compound **4** may thus provide a novel treatment option for colon cancer patients with an *A. baumannii* infection.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Merve Yildirim: Studied antimicrobial activity. **Elif Aksakal:** Studied the anticancer activity. **Fatma Betul Ozgeris:** Designed and evaluated the anticancer activity studies. **Bunyamin Ozgeris:** Designed the synthesis procedure and reviewed the manuscript. **Arzu Gormez:** Designed and evaluated the antibacterial activity studies.

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Secondary Metabolites in Fungi

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Abstract

Fungi are a biological family that includes multicellular and unicellular eukaryotic organisms in general. They are quite numerous and diverse. It is the second largest group of organisms on Earth after insects. Higher fungi (terrestrial fungi) currently constitute a large and efficiently untapped resource of new pharmacological crops with nutritional values are potentially powerful. Antioxidants prevent cellular damage caused by free radicals in living beings. It has been found that 36% of endophytic mushroom extracts with a high phenolic content show strong antioxidant effects. In one study, they reported that the antioxidant activities of the strains *Aspergillus* sp., *A. niger*, *A. peyronelii* and *Chaetomium* sp. ranged from 50% to 80% and that this antioxidant activity was due to their high phenolic content. In addition to the antioxidant properties of fungi, it has been shown as a result of research that they show antibacterial activity. Macrofungal β-glucans (β-1,6 glycosidic bonds and mainly β-1,3), which are known to have immunomodulatory and antitumor effects in cancer prevention, can be used in treatment strategies. At the same time, it has been shown in studies that these metabolites obtained from fungi cause fewer side effects when used in treatments, unlike chemical drugs. As a result of phytochemical research, the presence of flavonoids, anthraquinones, steroids, terpenoids, coumarins, phenols and tannins in fungal extracts in general was detected, while alkaloids and saponins were not found. It has been determined that the studies on mushrooms have gained versatile uses since they reveal secondary metabolite components with different biological activities in the structure of these group members.

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1. INTRODUCTION

Turkey has a rich floristic structure thanks to its phytogeographic location and climatic diversity. The presence of different aquatic environments such as sea, lake and stream and variable ecological zones at different altitudes allows both our fauna and flora to host a large number of endemic genera and species (Akyuz *et al*., 2010). Mushrooms are extremely abundant and globally diverse. It is the second largest group of organisms on Earth after insects. The latest estimates of the number of mushrooms in the world are about 1.5 million. The total number of described mushrooms of all types is currently 100,000 species. Fungi are, in general, the name of a biological realm that encompasses a large number of multicellular and unicellular eukaryotic creatures. Higher fungi (terrestrial fungi) are a vast and largely untapped source of powerful new pharmaceutical products, nutritional values, and potential constituents (Chen and Liu, 2017). Wild and cultivated mushrooms have been a part of human nutrition from the past to the present with their different and pleasant flavors (Erdoğan *et al*., 2017). Marine fungi, on the other hand, are an ecologically defined group rather than physiologically or taxonomically defined organisms. According to the 'classical' definition, which seems to be universally accepted in the scientific community, marine fungi are divided into two groups: obligate and

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facultative marine fungi (Rateb and Ebel, 2011). Endophytes have been described by various scientists as mutualists that colonize the aboveground parts of living plant tissues and do not cause disease symptoms. Endophytic fungi represent an important and quantitative component of fungal biodiversity and are known to influence plant diversity (Mishra *et al*., 2014).

Diseases have increased as technology has advanced, and mankind has begun to look for treatment methods in natural products grown in this rich geography (Duman *et al*., 2003). Mushrooms have been used in traditional medicine in many countries in addition to food with protein, fiber, vitamin and mineral richness (Erdoğan *et al*., 2017). It is very well known that plants and fungi have been used for therapeutic purposes since ancient times. Information about the importance and medicinal benefits of mushrooms has been found in ancient Egyptian hieroglyphs, Ancient Greek and Roman texts, Chinese medical texts. Mushrooms were also used in cultures such as the Maya, Aztecs and Inca for both medicinal purposes and religious rituals. Studies conducted today confirm the information about the traditional uses of medicinal mushrooms (Manzi *et al*., 2001).

The main reason why mushrooms can be used for therapeutic purposes is the secondary metabolites they contain. Secondary metabolites are organic molecules that do not participate in the normal growth and development of organisms. It plays an important role in increasing the viability of organisms and plant defences in the long term (Tiring *et al*., 2021). Fungi are extraordinary organisms capable of producing a wide variety of secondary metabolites. Fungi use secondary metabolites to chemically communicate with predators and competitors and manipulate host plants and animals, such as pathogenic fungi. It is well known that these components show antibacterial, antifungal, antiprotozoal and antiviral activities in some species of fungi. The antimicrobial effects of fungi are due to some phenolic compounds, purines, pyrimidines, quinones, terpenoids and phenyl propanoid derived antagonistic substances they synthesize, while their antioxidant potential is due to secondary metabolites such as phenolic compounds, polyketides, terpenes and steroids they contain. Not only terrestrial but also endophytic fungi show a large amount of antioxidant, antibacterial and phytochemical activity. The wide range of host endophytes means controlling several parasites, usually with the same product. Fungi are easy to grow in the laboratory and have great potential for the production of secondary metabolites (Mishra *et al*., 2014). Secondary metabolites cause the formation of free radicals called reactive oxygen species (ROS) as a by-product. ROS causes oxidative stress inside the cell, causing damage to the cell. Under oxidative stress conditions, antioxidant compounds synthesized by fungi come into play and protect the cell (İnci *et al*., 2019). Many studies on larvicidal activities have been conducted in recent years by researchers of the importance of secondary metabolites in plants and fungi for a better understanding of the function of antioxidant, antibacterial, larvicidal, anticancer, antidiabetic, anti-inflammatory, and phytochemical.

1.1. Biological Research Conducted on Fungi

1.1.1. Studies to Determine Antioxidant Activity

In addition to normal basic metabolic processes, the emergence of free radicals in organisms can also be caused by external factors such as heavy metals, air pollution, pesticides, some drugs, alcohol, smoking, and radiation exposure (Phaniendra *et al*., 2015). An excess of these free radicals in the body can cause many diseases such as cancer, cirrhosis, arteriosclerosis and rheumatoid arthritis and accelerate aging. Antioxidants prevent cellular damage caused by free radicals in living things (Nimse and Pal, 2015). It has been reported in many studies that there is a correlation between the phenolic Decients of mushrooms and their antioxidant content. Phenolic components include phenolic acids, tannins, oxidized polyphenols, flavonoids, lignans, hydroxybenzoic acids, stilbenes and hydroxycinnamic acids. These

components act as a stimulant in the synthesis of antioxidant molecules (Botterweck *et al*., 2000).

Yadav *et al*., (2014) wanted to elucidate 21 different endophytic fungal traces, antioxidant activity and total phenolic content (TPC) from *Eugenia jambolana* Lam. TPC was tested and performed with Folin-Ciocalteau reactive-based assay, while 2,2 diphenyl 1-picryhydrazil (DPPH) free radical sweeping, hydrogen peroxide sweep and reducing power methods were used to determine antioxidant activity. A significant positive correlation was found between antioxidant activity and TPC in mushroom extracts. 36% of endophytic mushroom extracts with high phenolic content were found to exhibit strong antioxidant activity. They also reported that the antioxidant activities of the strains of *Aspergillus* sp., *A. niger*, *A. peyronelii* and *Chaetomium* sp. ranged from 50% to 80%, and that this antioxidant activity was due to their high phenolic content. In addition, as a result of phytochemical analyzes carried out in addition to these studies, phenols, terpenes, alkaloids, saponins and flavonoids were determined as the main phytochemical components in all endophytes. The resulting data showed that metabolites produced by endophytes isolated from *E. jambolana* could be a potential source of new natural antioxidants.

In a different study, researchers isolated 21 endophytic fungi from five medicinal plants (*Trigonella foenum-graecum* L., *Calotropis procera* (Aiton) W. T. Aiton, *Vernonia amygdalina* Delile, *Euphorbia prostrata* Aiton and *Catharanthus roseus* (L.) G. Don) that grew in Sudan and evaluated the antioxidant capacity and total phenolic content (TPC) of ethyl acetate extracts. The phenolic content was classically tested by the Folin–Ciocalteu colorimetric method, while the antioxidant activity was determined by the DPPH radical scavenging assay. According to the data obtained in the study, *Aspergillus* sp. endophytic fungus obtained from *T. foenum-graecum* seeds showed the highest activity in terms of both TPC (89.9 ± 7.1 mg GAE/g) and antioxidant capacity (IC_{50}: (18.0 ± 0.1) mg/mL), and a linear relationship was found between TPC and antioxidant activity. Finally, in this study, it was emphasized that some endophytic fungi obtained from five Sudanese medicinal plants may be a natural source of medicine in terms of their antioxidant capacity (Khiralla *et al*., 2015).

In their study, Bharathidasan and Panneerselvam (2012) examined the antioxidant activity of ethanol extract of *Phomopsis amygdali* in vitro. 2,21 azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and DPPH methods were used to determine antioxidant activity. In addition, total phenolic and flavonoid secondary metabolite determinations of endophytes were also performed. The IC_{50} values of ethanolic *P. amygdali* extract were found to be 580.02 ± 0.511 µg/mL and 2030.25 ± 501 µg/mL, respectively, and it was found that they showed strong antioxidant activity.

In the study, which aimed to determine the antioxidant activities of 12 wild mushrooms (*Lactarius piperatus, Tricholoma anatolicum, Amanita caesarea, Lactarius deliciosus, Lactarius sanguifluus, Cantharellus cibarius, Hydnum repandum, Picoa lefebvrei, Ramaria aurea, Lactarius semisanguifluus, Craterellus cornucopioides, Laccaria laccata*) collected from different regions of Turkey, while total phenolic and flavonoid quantification experiments were carried out to determine the amount of secondary metabolites, DPPH method was used to determine antioxidant activity. The results showed that the total phenolic content of the mushrooms ranged from 575.10 to 2156.40 mg GAE/100 g DW, the total flavonoid content ranged from 103.01 to 346.53 mg CE/100 g DW, and the antioxidant activity was 525.32 mg CE/100 (Erdoğan *et al*., 2017).

Chandra and Arora (2009) isolated 113 fungi from soils collected from different regions of India in their study and aimed to determine the antioxidant activity of fungi by comparing them with ascorbic acid, which is known to have high antioxidant activity. In order to determine the antioxidant activity of mushroom extracts, they performed DPPH and reducing power

assays. In addition, Folins-Ciocalteau (FC) reagent was used for phenolic quantity determination. Of the 113 selected fungi, 51 reported that scavenging activity against the DPPH radical was present, while 32 also showed a reducing effect. At the same time, it was also revealed that some of these 51 fungal isolates had a more sweeping effect than ascorbic acid. With this study, researchers have proven that soil fungi can show antioxidant activity as well as plants and fungi.

Using ethanol extract of *Ganoderma lucidum*, the researchers aimed to determine antioxidant activity by conducting phenolic and flavonoid substance quantification experiments. In addition, the amount of ascorbic acid, which is known to be a very important antioxidant, has also been investigated. According to the obtained data, it was found that mushroom ethanol extract contains 42.41 ± 2.21 mg/g phenolic, 13.57 ± 1.57 mg/g flavone and 1.33 ± 0.12 mg/g ascorbic acid. According to the results of the study, it was emphasized that *G. lucidum* has a high level of antioxidant content and can be used as a food supplement (Rajasekaran and Kalaimagal, 2011). Kozarski *et al*. (2011), *Agaricus brasiliensis*, *A. bisporus*, *G. lucidum* and *Phellinus linteus* species have tried to detect the antioxidant activities of these fungi using DPPH radical superme, iron ions strengthening and reducing power methods. At the end of the study, it was determined that the antioxidant activities of *G. lucidum* and *P. linteus* species were equal, and *A. bisporus* species had the lowest antioxidant activity. *P. linteus* and 86.9%, *G. lucidum* of 94.5% with a value of DPPH free radical sweeping activities, ascorbic acid used as positive control (86.6%) is higher among these two species from the type of power was 30 times higher than the reductive bisporus.

In another study, researchers aimed to determine the antioxidant activity of oyster mushroom species. The methods of radical scavenging capacity of DPPH, β-carotene whitening activity, reducing power and lipid peroxidase inhibition were used in the study and, together with this, the species with the highest antioxidant activity was *Pleurotus flabellatus* has been found. *P. flabellatus* was followed *by P. florida* (16.6%), *P. eryngii* (15.6%), *P. cystidiosus* (14.6%) and *P. sajor-caju* (14.6%), respectively. In the same study, the species with the highest antioxidant potential was determined as *G. lucidum* with 30.1%, while the species with the lowest potential was *Flammulina velutipes* with 12.0% (Abdullah *et al*., 2012).

In their study, Nagda *et al*. (2017) aimed to determine antioxidant activity by obtaining methanolic extracts of endophytic fungi isolated from the leaves, stems and flower parts of the *Calotropis procera* plant. In the study, DPPH and reducing power methods were used to determine antioxidant activity, while simultaneously phenolic and flavone contents were examined. In the DPPH radical scavenging experiment of *Penicillium* species and *Aspergillus* species, the antioxidant activity of both isolates was 81.16% and 80.97%, respectively. According to the results of total phenolic quantity, both species were found to contain 9.16 µg/mg and 12.13 µg/mg phenolic respectively, while *Penicillium* species contained 130.50 µg/mg and *Aspergillus* species contained 94.91 µg/mg flavonoids. These metabolites, produced by endophytic fungi derived from *C. procera*, have been found to have very high antioxidant activity, but it is emphasized that more research needs to be done on them.

In a study investigating the relationship between total antioxidant capacity (TAC) and total phenolic content (TPC) by isolating 292 endophytic mushrooms from 29 traditional Chinese medicinal plants, it was found that there was a linear relationship between the antioxidant capacities and total phenolic contents of the endophytic mushrooms obtained. This led researchers to suggest that the antioxidant activities of endophytic fungi are due to the phenolics they contain. This study reports that endophytic fungal metabolites derived from medicinal plants may be the new natural source of antioxidants (Huang *et al*., 2007).

Sugiharto *et al*. (2016) conducted a study using ABTS, total phenolic, flavonoid, and tannin matter quantification methods to determine the antioxidant activities and components of

two filamentous mushrooms (*Rhizopus oryzae* and *Acremonium charticola*) isolated from Indonesian fermented dried cassava (gathot). As a result of the study, it was found that the ABTS radical sweeping activity of *A. charticola* was much higher than that of *R. oryzae* and ascorbic acid used as a control. In terms of phenolic and tannin component content, *A. charticola* was found to be higher, while the flavonoid content was found to be higher than *R. oryzae*. As a result, although both filamentous mushrooms showed antioxidant activity, it was found that *A. charticola* showed more antioxidant activity depending on the phenolic and tannin components it contained. In recent years, fungal antioxidant studies have focused on fungi that live on fruiting trees. Based on these studies, Lee *et al*. (2013a) extracted from solid cultures of 83 basidiomycetous mushrooms obtained from woody materials and evaluated their antioxidant activity. In the study, the fungi with the highest and lowest activity were determined by using DPPH and ABTS free radical sweep methods. According to the results of the experiment, 6 of the 83 mushrooms tested (*Stereum hirsutum*, *Cryptoporus volvatus, Gloeophyllum abietinum, Daedaleadickinsii, Pseudomerulius curtisii* and *G. trabeum*) were found to have high antioxidant activity. The basidiomycetous fungus with the highest activity was *Stereum sanguinolentum* with an IC_{50} value of 19.61 g/mL.

Arora, and Chandra (2011) have determined the antioxidant activities of *Penicillium citrinum, P. granulatum Aspergillus wentii* and *A. wentii* soil fungus species in various in vitro methods in their studies. According to the previous activity results, the fungi were grown in the environment where this species grew due to the high antioxidant activity of Czapek Dox agar. According to the results of the experiments, the antioxidant activity values of the mushrooms were found as *P. citrinum, P. granulatum* and *A. wentii,* respectively.

In a different study, the researchers aimed to determine the antioxidant activities of ethyl acetate extracts of endophytic fungi isolated from *Tragia involucrata* Linn. (Euphorbiaceae). DPPH radical removal, power reduction, and total antioxidant assay were used to evaluate antioxidant activity. Among the four isolated endophytic mushrooms (*Cladosporium* sp. *CGJ-D1*, *Cryptendoxyla hypophloia CGJ-D2*, *P.citrinum CGJ-C2* and *P. citrinum CGJ-C1*), *P. citrinum CGJ-C2* was found to have the highest antioxidant activity (Danagoudar *et al*., 2018).

In their study, Smith *et al*. (2015) aimed to investigate the antioxidant capacity of ten filamentous mushroom species grown in sunken bottle cultures. β-carotene/linoleic acid fungi have been reported to show high antioxidant activity in terms of bleaching, radical excretion, reduction of metal ions, and chelating abilities against iron ions. Different extraction methods have been shown to influence antioxidant activities with their effects on biologically active compounds produced in mushroom micelle. Each mushroom's methanolic extract is typically found to be better at antioxidant properties. Its phenolic content was in the range of 0.44–9.33 mg/g, the flavonoid content was in the range of 0.02–3.90 mg/g, and the condensed tannin content was in the range of 1.77–18.83 mg/g. The total phenol content of each extract was found to be positively correlated with the overall antioxidant capacity. In the study, it was reported that submerged culture is an effective method for the synthesis of metabolites exhibiting antioxidant activity of *Pleurotus* spp., *Monascus purpureus, Grifola frondosa*, *Trametes versicolor* and *Lentinula edodes* fungal species.

In their study, Soyuçok *et al*. (2022), the antioxidant activity of ethanolic extracts of *Suillus granulatus* type fungi was determined. The DPPH method was used to determine antioxidant activity, while high-pressure liquid chromatography (HPLC) was used to determine the phenolic content. Researchers have found that *S. granulatus* fungal metabolites exhibit antioxidant activity and that ellagic acid, caffeic acid and gentisic acid are the most common phenolic components they contain.

1.1.2. Antimicrobial Studies of Mushroom Extracts

There are many studies that the myceliums of fungi, their bodies and hats are sources of antibiotics with antibacterial effects. Barros *et al*. (2007) reported that *Lactarius deliciosus* had stronger antimicrobial activity than *Sarcodon imbricatus* and *Tricholoma portentosum*. The study also suggested that total phenol and flavonoid levels were directly related to antimicrobial activity. Stanley *et al*. (2018) found that ethanolic extracts of *Pleurotus tuber-regium* significantly affected the growth of *Candida albicans* (7 ± 0.1 mm), *Klebsiella* spp. (21 ± 0.2 mm), *Staphylococcus aureus* (13 ± 0.2 mm) and *Bacillus* spp. (15 ± 0.4 mm). The researchers also reported that *Pseudomonas aeruginosa, Escherichia coli,* and *Streptococcus* spp. strains showed resistance to the ethanol extract of the fungus.

In their study, Peláez *et al*. (1998) aimed to isolate 152 endophytic fungi from 9 plants growing in gypsum and saline soils of Spain and measure their antibacterial activity. A total of 187 strains from 136 species were tested to induce antibacterial activities using a group of bacteria, some of which are of clinical importance. As a result of the research, it was found that even if the isolates are of the same species, they produce metabolites with different antimicrobial activities.

Skalicka-Wozniak *et al*. (2012) reported that the D-glucose component they extracted from *G. lucidum* polysaccharide showed high activity in inhibiting bacterial growth. In the studies of Chowdhury *et al*. (2015), three different mushroom species (*Hypsizygus tessellatus*, *L. edodes* and *P. ostreatus*) aimed to determine their antimicrobial activity. As a result of the study, it was determined that *L. edodes* had the best antimicrobial activity, while *P. ostreatus* showed activity against many bacterial strains. In addition, the fungi *P. ostreatus* and *H. tessellatus* have been reported to show the strongest activity against the *B. subtilis* strain.

In another study, researchers aimed to test their antimicrobial activity by isolating endophytic fungi from five *Garcinia* plants (*G. scortechinii* King*, G. dulcis* (Roxb.) Kurz*, G. nigrolineata* Planch. ex T. Anders*., G. atroviridis* Griff. ex T. Anderson *and G. mangostana* L.). The antimicrobial activities of fermentation mediums obtained from 377 endophytic fungi isolated from the leaf areas of plants were tested by diffusion agar method. After obtaining minimum inhibitory concentrations (MICs) of fungal ethyl acetate extracts, they have been found to exert antimicrobial action against microorganisms such as methicillin-resistant *S. aureus* and *C. albicans*. In this study, it was concluded that some endophytes of the *Garcinia* plant may be a potential source of antibacterial agents (Phongpaichit *et al*., 2006).

Al-Faqeeh *et al*. (2019) have shown that methanol extract from *Hypsizygus ulmarius* (bull) fungus has the highest activity against *Enterococcus faecalis, Proteus mirabilis, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. In the study, the ethyl acetate fraction of this fungus, *P. aeruginosa* and *K. pneumoniae* bacteria; petroleum ether extract was reported to inhibit the growth of *P. aeruginosa, E. coli, K. pneumoniae* and *P.mirabilis* strains.

In another study, *Amanita rubescens*, *Russula cyanoxantha*, *Lactarius piperatus*. and *C. cibarius* species have reported that acetone and methanol extracts show strong antibacterial effects. According to the test results, acetone extract of *L. piperatus* species showed the highest antimicrobial activity against *E. faecalis* with a MIC value of 1.25 mg/mL, while the antimicrobial activities of both acetone and methanol extracts of *A. rubescens*, *C. cibarius* and *R. cyanoxantha* species were found to be very close (Kosanić *et al*., 2013).

Marcellano *et al*. (2017) aimed to test their antimicrobial activity by isolating pharmacologically important endophytic fungi from the medicinal tree *Cinnamomum mercadoi* S. Vidal. Then, the morphological properties of the isolated mushrooms were determined and they were separated according to these characteristics. Initially, an agar plug diffusion test was used to examine their antibacterial activity against *S. aureus, P. aeruginosa, E. coli* and *Bacillus*

cereus. To evaluate the screening results, the Minimum inhibition activity (MIC) microdilution method was deemed appropriate. As a result of the study, it was found that all endophytes showed antibacterial effects in at least one of the bacterial strains used for testing. In addition to these, another screening was carried out with 4 endophytes, and only two of them were found to inhibit the growth of all bacteria used. The endophytic fungus with the highest antibacterial activity has been reported to be *Fusarium* sp. In a study investigating the effect of aqueous and ethanolic extracts of *A. bisporus* type fungus (50 mg/mL) against the growth of *Salmonella typhi* and *S. aureus* strains, it was found that aqueous fungus extracts did not affect either bacterial strain, but ethanol fungal extracts acted with very high activity on *S. typhi* (9 mm) and *S. aureus* (15 mm) strains (Hussein *et al*., 2018). Waqas *et al*. (2018) reported that *A. bisporus* ethyl acetate extract inhibited the growth of *Drechslera australiensis* fungi by 50% and inhibited the growth of *Alternaria alternata* and *Fusarium oxysporum* species by 44%.

Ramesh and Pattar (2010) investigated whether 4 different fungi (*Lycoperdon perlatum, Pleurotus pulmonarius, Marasmius oreades* and *Clavaria vermicularis*) had antibacterial efficacy against *E. coli*. According to the results of the study, *L. perlatum* (24.0 mm) exhibited the highest in vitro antibacterial activity, followed by *P. pulmonarius, M. oreades* and *C. vermicularis* species respectively.

In the study of *Volvopluteus gloiocephalus* (DC.) Vizzini and *Clitocybe subconnexa* Murrill mushrooms, they found that they had more antimicrobial activity than ampicillin and streptomy, which are antibiotics used as positive controls. In this study, they also noted that *S. aureus* was the most susceptible type of bacteria to phenolic extract of *V. gloiocephalus* fungus, and that phenolic extract of *V. gloiocephalus* showed better antifungal activity in digested samples. Based on their results, the researchers also suggested that all samples exhibited superior antifungal activity compared to ketoconazole and bifonazole (Heleno *et al*., 2015).

Getha *et al*. (2009) evaluated the antibacterial activity of different fungi belonging to the Polyporales order against *Bacillus subtilis*, *Rhodotorula glutinis*, *Saccharomyces cerevisiae* and *S. aureus*. As a result of the study, it was seen that the extracts of *Ganoderma* genus with the highest antimicrobial activity were the mushrooms with 62.5%, followed by the extracts obtained from the *Rigidoporus* genus with 27.2%. They reported that the fungus with the lowest antibacterial activity was extracts obtained from species belonging to the genus *P. linteus* with 7.8%. In the studies of Moglad and Saadabi (2012), 7 different wild mushroom species *(Coprinus comatus, Agaricus arvensis, Agaricus silvicola*, *Agaricus porphyrocephalus*, *A. bisporus*, *A. bernardii* and *Lepiota cristata* sp.) by extracting extracts of water, ethanol and petroleum ether, they investigated the antimicrobial activity of each against a different strain of bacteria. Researchers have concluded that ethanol is the ideal solvent for obtaining antibacterial compounds. They also found that when compared to other mushrooms in the study, the ethanolic *A. porphyrocephalus* extract showed the highest antimicrobial activity against all microorganisms and the most effect was on the *E. coli* strain.

In another study, 4 different extracts (petroleum ether, ethyl acetate, methanol and water) of *Pleurotus eous* mushroom species were obtained and their antibacterial activities against *B. cereus, P. aeruginosa, K. pneumoniae, E. coli, S. aureus* and *B. subtilis* were tested using MIC method. In the results, it was determined that petroleum ether extracts had the highest MIC values. The MIC values of petroleum ether extracts of the species were 3.1 µg/mL (*E. coli*), 3.1 µg/mL (*B. subtilis*), 4.2 µg/mL (*B. cereus*), 4.4µg/mL (*S. aureus*), 4.4 µg/mL (*K. pneumoniae*) and 8.8 µg/mL (*P. aeruginosa*) (Suseem and Saral, 2013).

Casaril *et al*. (2011), in a study evaluating the effects of different media on the antibacterial properties of four *L. edodes* isolates (FV11(Le1), UFV52, UFV62 and UFV63), reported that although all *L. edodes* isolates were exposed to different environments and were extracted by different methods, they all had antibacterial activity in various ways.

In their study, Kosanić *et al*. (2012) determined the in vitro antimicrobial activities of acetone and methanol extracts of *Boletus aestivalis*, *B. edulis* and *Leccinum carpini* species using the MIC method. According to the results obtained, it was determined that the acetone extract of the *Leccinum carpini* species had the highest antimicrobial activity against *Enterococcus faecalis* (gram positive bacteria) with an MIC value of 1.25 mg/mL. In the study, acetone and methanol extracts of *B. aestivalis* and *B. edulis* species were reported to have almost similar antibacterial activity.

1.1.3. Larvicidal Effects of Mushroom Extracts

Mohanty and Prakash (2009) investigated the effects of secondary metabolites of the *Chrysosporium lobatum* fungus obtained by filtration from Sabouraud dextrose (SDB) and chitin juice (CB) on mosquito larvae (*An. stephensi and Culex quinquefasciatus*) and found that *An. stephensi* species are less sensitive to secondary metabolites obtained from SDB and cb than *C. quinquefasciatus*, and the $LC_{50}$and LC_{90} values of the first instards of both species are lower than other 2 and 3 instards reported. The researchers also found that the *An. stephensi and C. quinquefasciatus* populations had mortality values of 56.3% and 66.42%, respectively, and that the mortality rate in CB was higher compared to ESB. According to this result, they suggested that the higher mortality in CB may be due to proteases contained in secondary metabolites.

In another study, researchers isolated the endophytic fungus *Aspergillus tamarii* from *Opuntia ficus-indica* (L.) Mill. and evaluated the larvasidal effects of this mushroom species on mosquitoes *Aedes aegypti* and *Culex quinquefasciatus*. A protocol consisting of five different ethyl acetate extract concentrations and control group (DMSO and distilled water) was developed, while LC_{50} and LC_{90} lethality values were calculated. As a result of the study, the researchers found that *A. tamarii* endophytic mushroom extracts showed a higher larvasidal effect on *Ae. aegypti* mosquitoes. In addition, they emphasized that both mosquito species had very low LC_{50} and LC_{90} values and exhibited excellent larvasidal activity (Baskar *et al*., 2020).

Sathiyanathan and Umarajan (2019) aimed to evaluate the larvicidal activity of ethyl acetate extracts of 3 different endophytic fungi (*Aspergillus, Penicillium* and *Fusarium*) isolated from the leaves of six different plant species (*Tagetes erecta* L.*, Andrographis paniculata* (Burm.f.) Nees*, Vitex negundo* L.*, Mentha* × *piperita* L.*, Ocimum tenuiflorum* L. and *Azadirachta indica* A. Juss.). The study reported a significant increase in mortality percentages in direct proportion to concentration, with the highest larvasidal activity exhibited by *Fusarium* and *Aspergillus* strains isolated from *V. negundo* leaves with values of 90.47% and 85.71% at 20 ppm. Abutaha *et al*. (2015) recorded the changes by applying raw extracts to the larvae of the third instar level in their study investigating the effects of the *Cochliobolus spicifer* endophytic fungus on the *Cx. pipiens* and *Aedes caspius* (Pallas) larvae, which they isolated from the date palm tree in Saudi Arabia. As a result of the study, the researchers found that histological changes occurred in the digestive system of the larvae and reported that the raw extracts killed all of the larvae at a concentration of 300 ppm. In addition, they emphasized that *C. spicifer* mushroom extract can be a natural and harmless method that can be used to balance the populations of *Ae. caspius* and *Cx. pipiens*.

In a different study, the larvasidal activities of *Aspergillus flavus*, *Penicillium verrucosum*, *Fusarium sporotrichioides*, *A. parasiticus* and *A. niger* endophytic fungi on *An. stephensi and C. quinquefasciatus* larvae were evaluated. According to the data obtained after 24-hour administration, *A. flavus* was reported to be the endophytic fungus with the highest larvasidal activity with LC_{50} mortality values of 9.54 and 10.98 against *An. stephensi* and *C. quinquefasciatus* larvae, respectively. In addition, they emphasized that after the 48-hour observation period, the mortality rate increased in direct proportion to the duration (Maurya *et al*., 2011).

Bücker *et al*. (2013) investigated the effects of ethyl acetate mycelium extracts of *Pestalotiopsis virgulata* and *Pycnoporus sanguineus* endophytic fungi on *Aedes aegypti* and *Anopheles* larvae in the third instar phase at five different concentrations. In the results, the researchers found that *P. virgulata* extracts had a higher mortality effect than *P. sanguineus* with LC_{50} values of 101.8 and 16.3 ppm, respectively. Unlike other studies, in this study, the researchers were able to extract *Podospora* sp. from the *Laggera alata* S. Moore (Asteraceae) plant. By isolating the endophytic fungus, they evaluated the larvicidal effects of 3 compounds of this endophytic fungus (sterigmatocycin (1) and sekosterigmatocystine (2) and 13-hydroxyversicolorycin B (3)) against the third instar larvae of *Anopheles gambiae*. According to the data obtained, the compound with the highest larvicidal activity was 13.3 ppm LC_{50} and 73.5 ppm LC_{90} values 1. It has been reported to be compounded. At the end of 24 hours of application time 1. It was also determined that the mortality rate of the compound was 95%. In addition to these, 1st place in the activity ranking Compound 3. That the compound follows and 2. They stressed that the LC values were not determined because the activity of the compound was too low (Matasyoh *et al*., 2011).

Tian *et al*. (2016) suggested that endophytic fungi could be a source of pesticides, and in response to these considerations, 7 fractions of ethyl acetate extracts of the inflammatory fungus of the Asian tiger mosquito *Aedes albopictus* larvae (palmarumycins C8, C12, C15 and B6 and diepoxins γ, δ and ζ) were applied. According to the data obtained, the researchers reported that chlorine-containing palmarumycins C8 and B6 were the compounds with the highest larvicidal activity with values of 8.83 and 11.51 µg/mL LC_{50}, respectively. As a result of the study, they emphasized that compounds obtained from *Berkleasmium* endophytic fungus could be a promising source of pesticides. In another study that sought natural and safe pesticides, the effects of 11 dibenzo-×-pyrons (1-11) of *Ae. aegypti* on fourth instellar larvae caused by the fraction of *Hyalodendriella* sp. *Ponipodef12* isolated from poplar tree (*Populus deltoides* W. Bartram ex Marshall *α P. nigra* L. Hybrid) were evaluated. In the results, it was determined that the 2nd-7th compounds out of 11 compounds obtained had a Decently high larvicidal activity (IC_{50} between 7.21 and 120.81 µg mL-1). Among the 6 compounds with high activity, the compound with the strongest effect was reported to be the sixth compound (penecilliumolide D) with an IC_{50} value of 7.21 µg/mL. The researchers emphasized with their study that dibenzo-α-pyrones can be a very powerful source of drugs that can be used against larvae (Mao *et al*., 2017).

Salunkhe *et al*. (2011) aimed to investigate the effects of silver nanoparticles (AgNPs) obtained from the *Cochliobolus lunatus* fungus on the larvae of *Ae. aegypti* and *An. stephensi* mosquito species, which cause diseases in living beings, in their study. Six at different concentrations LC_{50} and LC_{90} values of 1.17 2.99 ppm AgNPs prepared *An. stephensi* showed the highest activity against larvae of mosquitoes and the third and fourth have found that mortality increased directly proportional to the concentration larvicidal. However, they reported that AgNPs showed strong activity on *Ae. aegypti* larvae, although not as strong as *An. stephensi* (LC_{50} 1.29 and LC_{90} 3.08ppm). The researchers also report that this is the first study to evaluate the larvicide activity of silver nanoparticles isolated from the *C. lunatus* fungus.

Seetharaman *et al*. (2017) isolated the *Penicillium oxalicum LA-1* endophytic fungus from *Limonia acidissima* L. plant and investigated the larvicidal activity of hamisonin (HMSN) compound found in this fungus in their study in order to detect new bio-compounds with larvicidal activity. Looking at the LC_{50} (1.779 and 3.031 ppm) and LC_{90} (7.685 and 28.498 ppm) values of HMSN compound applied on the third and fourth instar larvae of *C. quinquefasciatus* mosquito, they found that this compound has high larvicidal activity.

1.1.4. Anticancer Studies of Mushroom Extracts

One of the extremely serious diseases with a high mortality rate is cancer. The constant increase in the number of cancer patients all over the world has made this disease a global problem, and therefore finding a cure for this disease has become a priority of the scientific world. Cancer drugs used to treat cancer can cause unwanted side effects in some cases and miss their target. For such reasons, researchers have begun to look for different treatment strategies in order to minimize side effects and get better results. Improved phytochemical and pharmacological studies in recent years have shown that one of the most important bioactive compounds of fungi are polysaccharides. Known to have immunomodulatory and antitumor effects in the Prevention of cancer Makrofungal β-glucans (β-1,6 glycosidic bonds and mainly β-1,3) can be used in treatment strategies. At the same time, it has been shown in studies that these metabolites obtained from fungi cause fewer side effects when used in treatments, unlike chemical drugs. Therefore, they can be used to support anti-cancer treatment and to produce alternative medicines (Öztürk and Atila, 2021).

Çöl *et al*. (2017) used prostate cancer cell line (PC-3) and cervical cancer cell lines (HeLA) to investigate the anticancer effects of both mycelium and dried mushroom extracts of *Schizophyllum commune* species. As a result of the study, it was found that mycelium and dried mushroom extracts showed a high cytotoxic effect on PC-3 and HeLa cell lines.

Lentinan, a type of polysaccharide isolated and purified from *L. edodes*, is known to be very important in increasing the effectiveness of cancer treatment. Zhang *et al*. (2015) stated that *L. edodes* polysaccharide (JLNT) showed antitumor activity against sarcoma-180 tumor cells and human colorectal cancer lines in their studies. In addition, studies have emphasized that the antitumor effect of polysaccharides is related to the content and molecular weight of monosaccharides. In another study, researchers also reported that *L. edodes* can be a natural tumor suppressor and significantly inhibit the development of cancer cells (Finimundy *et al*., 2018).

Lectins cause the destruction of cancer cells and the proliferation of active lymphocytes (Öztürk and Atila, 2021). Sałata *et al*. (2018) suggested that many bioactive components in *P. ostreatus,* especially ß-glucan and lectin, can be used as auxiliary substances in the treatment of cancer and in chemotherapy and radiotherapy of various types of cancer, especially in advanced stage cancers. In a different study, researchers also reported that enzymes obtained from *Pleurotus* species have beneficial and curative effects on health in general, and *P. djamor* ribonuclease inhibits the proliferation of lung and breast cancer cells (Wu *et al*., 2010).

Sohretoglu and Huang (2018), *G. lucidum* of polysaccharides by improving the immune system, cell proliferation, and metastasis of malignant tumors by blocking the tumor showed anticancer activity by preventing new blood vessels from forming. In another study conducted on *G. lucidum*, it was aimed to determine the anticancer effect of Ganoderic acid-A obtained from the plant on human hepatocellular carcinoma cell lines. For this purpose, in three different time periods, 24, 48 and 72 hours, and in different doses (50, 100, 150, 200, 250 and 300 µmol/L) have been applied on cell lines in their results, they found that the development of HepG2 and SMMC7721 human hepatocellular carcinoma cell lines was significantly inhibited after 48 hours of treatment with a dose of 100 µmol/L. It was also reported that a 72-hour exposure to ganoderic acid-A at 75 µmol/L had a similar effect, significantly inhibited the growth of both cells, and the ideal treatment in the results was 100 and 75 µmol/L (Wang *et al*., 2017).

Two very different studies, researchers in one type of fungus *Macrolepiota procera* colon cancer, LS174 and A549 cell lines in the other cytotoxic against tumor cells in two studies to determine the effect of extracts inhibits the growth of mprocera worked and reported. In the

second study, in addition to the *M. procera* species, HeLa cell lines were used against *L. deliciosus* fungus has been tested. In the results obtained, it was found that *L. deliciosus* significantly inhibits the growth of tumor cells (Xu *et al*., 2012; Kosanić *et al*., 2016).

Pleuran is a β-glucan compound obtained from *Pleurotus ostreatus* extract. Several studies have reported that this compound has the activity of inhibiting the proliferation of various cells such as breast cancer cells MCF-7, prostate cancer cells PC-3, and colon cancer cells HT-29 (Öztürk and Atila, 2021). Tong *et al*. (2009), conducted and obtained POPS-1, a water-soluble polysaccharide, from *P. ostreatus* activities. At the end of the study POPS-1 in vitro antitumor activity against HeLa tumor cells with high and 5-florourasil is suitable for the development of antitumor agents that may be less toxic than the source emphasized.

Cordyceps fungi have been used in traditional Chinese medicine for many years to treat many diseases, including cancer. In a study, the effects of ethanol extract of *C. militaris* Link type fungus on RKO cell line, which is a derivative of human colorectal cancer, were investigated. As a result of the study, it was reported that cell lines could not resist ethanolic *C. militaris* extracts and tumor formation was prevented (Öztürk and Atila, 2021). Wada *et al*. (2017), on the other hand, in their study on *C. militaris* extracts, they reported that these extracts contain a large amount of clicitol and that this metabolite can prevent the formation of cancer cells. In another study conducted on the *Militaris* species, Tomonobu *et al*. (2020), reported that xylitol from *C. militaris* has antitumor activity, and xylitol effectively sensitizes cancer cells to chemotherapy drugs. In the study, it was emphasized that the chemotherapy strategy applied with xylitol in cancer patients gives positive results and may be an important strategy that can be used to improve the treatment results of cancer patients.

Jin *et al*. (2018) conducted and suggested that they suggested that a metabolite called Cordyceps obtained from the *C. militaris* species regulates apoptosis, stops the cell cycle and prevents the development of a tumor by targeting cancer stem cells (CSC). In the study, they also suggested that Cordyceps could be a source of drugs to prevent the spread of the tumor to the environment by suppressing tumor metastasis.

In a different study, researchers 0.1 and 5 mg/mL of different concentrations ranging from *G. lucidum* triterpene extract (GLT) cell viability, invasion, proliferation, and to investigate how the two affect that result in human prostate cancer cells (DU-145) have used. The researchers found in the results that a high dose of GLT (2 mg/mL) inhibits cell viability in a dose- and time-dependent manner by increasing matrix metalloproteases, and GLT induces apoptosis of DU-145 cells. As a result of the study, it was reported that GLT affects cancer cells by different mechanisms and may have potential therapeutic uses in cancer prevention and treatment (Qu *et al*.,2017).

Zhang *et al*. (2014) reported that polysaccharides from *A. bisporus* have strong immunostimulatory and antitumor bioactivity in vivo and in vitro. In another study, researchers aimed to investigate the effects of *G. lucidum* polysaccharides (GLP) on prostate cancer (PCa) PC-3 cells and compared the dose- and time-dependent viability of GLP-treated PC-3 cells with the control group. They determined that the viability of PC-3 cells treated with GLP at a concentration of 10 mg/mL decreased by 5.4% ± 5.4% in 24 hours, 36.4% ± in 48 hours, 3.9 and 21.5% ± 2.8 in 72 hours. In the same study, they also found that GLPs showed similar activity against PCa DU145 and LNCaP prostate cancer cells and inhibited cell viability (Wu *et al*., 2018).

Mei *et al*. (2015) investigated the effects of PLPS1 and PLPS-2 polysaccharides isolated from the micelles of *Phellinus linteus* on sarcoma-180 tumor cells. As a result of the study, they found that PLPS1 exerts a strong inhibitory effect on cell proliferation, while PLPS-2 does not. They also suggested that this result is due to Deconstructive differences between them.

Hispolone is one of the phenolic components that can be isolated from *P.linteus*, it also has the ability to kill bladder and breast cancer cells (Öztürk and Atila, 2021). Huang *et al*. (2011) in their study, they reported that when *P.linteus* micellar culture extract (MCPL-7) was used regularly for 8 weeks, a significant decrease in tumor size and an increase in T cells were observed.

Five different fungal strains (*Hericium erinaceus*, *P. ostreatus*, *L. edodes*, *G. lucidum* and *V. volvacea*) In the study investigating the effects of HT-29 on the colon cancer cell line, a dose of 200 µg/L was applied to the cell lines and it was found that *P. ostreatus* species showed the highest anticancer activity with a rate of 84%. It has been reported that this species is followed by *V. volvacea* with 83%, *L.edodes* with 68%, *H. erinaceus* with 66% and *G. lucidum* with 29%, respectively. In, the study also investigated the effects of the species on the development of the H-1299 lung carcinoma cell line, and the results showed that *H. erinaceus* inhibited the development of this cell line by 72%, *P. ostreatus* by 61% and *G. lucidum* by 24% (Sharif *et al*., 2018).

Huo *et al*. (2017) used 12 different cancer cell lines (SK-MEL-28, CoLo205, Raji , HCT-116, Lewis, Hep3B2.1-7,MDAMB-453, K562, HeLa, B16F10, NCI-H460 and A549) to investigate the dose-dependent anticancer effects of two polysaccharides (AECS1 and AECS2) derived from water extracts of the *Cordyceps sinensis* (AECS) type fungus. As a result of the study, they reported that IC_{50} values of AECS1 ranged between 0.02-3.2 mg/mL and AECS2 ranged between 0.31- 4.08 mg/mL. The IC_{50} values of AECS1 ranged between 0.02-3.2 mg/mL and AECS2 ranged between 0.31-4.08 mg/mL. According to the data obtained, it was found that both AECS1 and AECS2 had growth-inhibiting effects on the tested cell lines, but AECS1 showed higher anticancer activity. In addition, as part of the test, a treatment strategy with a combination of DDP+AECS2 was applied to mice with Lewis tumor cells, and it was found that recovery was significantly better in mice treated with a combination of AECS2.

1.2. Pharmacological Studies That Have Been Conducted on Fungi

1.2.1. Studies of Anti-Inflammatory Activity

Cheng *et al*. (2016) Isolated sulfate-free (PS) and sulfate-free (SPS) polysaccharides from mycelia of *Antrodia cinnamomea* culture fungus and used lipopolysaccharide (LPS)-induced RAW264.7 macrophages to investigate the anti-inflammatory effects of these fungal polysaccharides. In the data obtained, it was found that SPS inhibits the release of interleukin-6 (IL-6) and tumor necrosis factor-a (TNF-a) more strongly than PS. At the end of the study, the researchers reported the availability of sulfated polysaccharides obtained from the *A. cinnamomea* fungus in anti-inflammatory studies by adjusting the sulfate degree.

In another study, the fungi *Aspergillus terreus* cultures from four aromatic butenolid asperimid endophytic solid A–D (1-4) and previously known analog butirolakton-1 (5)'s Lipopolisakkarit (LPS) stimulated raw 264.7 cells with blocking the production of nitric oxide in anti-inflammatory effects tested. As a result of the study, it was reported that asperimide A–D 3 (IC_{50} 0.78±0.06 µM) and asperimide A–D 4 (IC_{50} 1.26±0.11 µM) compounds exhibited strong anti-inflammatory activity compared to other compounds (Liao *et al*., 2018).

Liu *et al*. (2019), in their study *Diaporthe* sp. GZU-1021 fungus obtained from 4 new composite (biatriosporin N (7) and diaportikalazin a-C (1-3)) and the previously known compound 6 (4-6 and 8-10) anti-inflammatory activity and are intended to assess. For this purpose, they followed the inhibition of nitric oxide production by treating lipopolysaccharide-derived microglial cells (RAW264.7 cells) with isolated compounds. As a result of the study, 5th-10th 1.94 µm and IC_{50} values of the compounds to 16.5 than indometasin has changed between used as positive control (IC_{50} = 29.7 µM) stronger anti-inflammatory activity showed that it identified.

Researchers isolated seven compounds (4 new compounds, amestolcolides A-D, and three previously known compounds) from the *Talaromyces mestolkiae*-YX1 endophytic fungus isolated from wheat solid culture in a study looking for new anti-inflammatory compounds.They then used these seven compounds to inhibit nitric oxide (NO) production in lipopolysaccharide-induced RAW264.7 cells. According to the results obtained, they reported that amestolcolide B exhibited a strong anti-inflammatory effect with an IC_{50} value of 1.6 ± 0.1 µM (Chen *et al*., 2018).

Khayat *et al*. (2019) Isolated *Fusarium* sp. endophytic fungus from the roots of *Mentha longifolia* (L.) Huds. (Labiatae) plant grown in Saudi Arabia. Within the scope of the study, they aimed to evaluate the anti-inflammatory activity of 5 different metabolites obtained from *Fusarium* sp. fungus due to the inhibition of 5 lipoxygenase (5-LOX) enzymes. in the results, they reported that the first and second compounds showed an anti-inflammatory activity close to indomethacin (IC_{50} 1.17 µM) used as a control with IC_{50} values of 3.61 and 2.45 µm, respectively.

Liu *et al*. (2018) isolated from mangrove fungus *Aspergillus terreus* be ostinoid endophytic in the work of 1,2-dehidro-terredehidroaustin (1) and two known meroterpenoid (2 and 3) of the compounds lipopolisakkarit (LPS) induced by RAW246.7 mouse macrophages nitric oxide (no) to inhibit the production of the power is being explored. According to the data obtained, 1. The compound has been reported to be very weak in inhibiting nitric oxide (NO) production (IC_{50} value 42.3 µM).

In a different study, a marine fungus *Penicillium* sp. JF-55 than isolated compound 3 (penstirilpiron (1), anhidrofulvik Acid (2) and sitromiset (3)) anti-inflammatory activity tested. among the three compounds, they found that only the first compound inhibits the production of iNOS and COX-2, indirectly inhibiting the production of NO and PGE2 Decently. It has also been associated with compound 1's reduction of TNF- α and IL1ß production, suppression of phosphorylation and degradation of Ik-B α, NF-kB nuclear translocation, and NF-kB DNA binding activity. However, researchers have reported that using tin protoporphyrin (SnPP), compound 1 triggers the production of HO-1 and inhibits the expression of pro-inflammatory mediators via NFkB (Lee *et al*., 2013b).

Kim *et al*. (2013) isolated two indole alkaloids, neoechinulin A and B, from the marine fungus *Eurotium* sp., in their study and aimed to evaluate the anti-inflammatory effects of these compounds on lipopolysaccharide (LPS)-induced RAW264.7 macrophages. As a result of the study, the researchers Neoesinulin A (1) ‘s without affecting cell viability at concentrations ranging from 12.5 µM to 100 µm nitric oxide (no), prostaglandin E2 (PGE2) and inhibit the production of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) enzymes have been shown to suppress their expression. In addition, they revealed that Neoechinulin A exerts an anti-inflammatory effect through inhibition of nuclear factor-kappa B (NF-kB) and p38 mitogen-activated protein kinase (MAPK) pathways. However, although Neoechinulin B inhibits NO production at lower doses, they reported that it affects cell viability at a concentration of 25 µm.

1.2.2. Antidiabetic Effects of Fungi

When a person's blood sugar rises, the metabolic disease that occurs when the body's cells cannot respond to insulin or cannot produce insulin at all is called diabetes (Öztürk and Atila, 2021). Zhang *et al*. (2018) aimed to treat streptozotocin-induced diabetic rats using metabolites of *P. ostreatus* type fungus. As a result of the study, they emphasized that these metabolites reduce the levels of hyperlipidemia and hyperglycemia, regulate insulin resistance and increase the storage of glycogen.

Ficus religiosa L. obtained from a plant called antidiabetic effects were investigated in a study where endophytic of fungi, mushroom extracts endophytic α-amylase inhibition assay, α-glucosidase inhibition assay analysis test with 3 different diffusions and glucose were analyzed. As a result of the study, the researchers endophytic fungi, α-amylase inhibition assay (91 ± 0.06%) and α-glucosidase inhibition Tests (42 ± 0.01%) showed a high percentage inhibition, and these colonies possess antidiabetic activity have reported that (Tiwari *et al*., 2017).

Shehata *et al*. (2010) reported that *P. ostreatus* and *A. bisporus* species exhibited significant hypoglycemic activity in their studies, and the amount of liver glycogen and glucokinase production of living beings increased significantly. Kumar *et al*. (2018), in their studies, they determined that the metabolites of *A. bisporus* fungus have an antidiabetic effect of 78.85% in vitro at a dose of 2 mg/mL. Balakrishnan and Loganayagi (2018), evaluated the hypoglycemic effect of ethanolic and methanolic extracts of the *A. bisporus* fungus in rats stimulated with alloxan and found that the best results were obtained with ethanolic extract. Alloxane stimulated with serum glucose in the diabetic group compared to the normal group, marker enzymes (AST, ALT, ALP, lactate dehydrogenase (LDH)) and a significant increase in lipid levels, serum protein and liver glycogen concentration was observed a significant decrease in reported.

By isolating 22 endophytic fungi from two medicinal plants known to have antidiabetic effects (*Momordica charantia* and *Trigonella foenum-graceum*), the researchers aimed to investigate whether ethyl acetate extracts of these mushrooms could also have an antidiabetic effect. For this research, the Deciency of inhibition of α-amylase, α-glucosidase and aldose reductase Inhibitors was tested and it was determined that only 9 isolates out of 22 endophytic mushroom extracts could inhibit α- amylase and α- glucosidase enzyme inhibitors. It was determined that the crude extracts of fungal isolates PTFL005 and PTFL006 showed a significant α-amylase inhibitory effect with IC_{50} values of 15.48 and 13.48µg / mL, respectively. PTFL006 and PTFL011 fungal isolates were found to have strong α-glucosidase inhibitory activities close to acarbose (6.53µg/mL) used as a control with IC_{50} values of 17.37 and 10.71µg/mL, respectively. Finally, it was emphasized that only PMCF003 isolate showed reasonably better inhibition for aldose reductase with an IC_{50} value of 40µg/mL compared to standard quercetin (15µg/mL) (Pavithra *et al*., 2014).

Ng *et al*. (2015) tested the hypoglycemic and antidiabetic activities of the fungus by administering aqueous extracts of the *P.sajor-caju* type fungus to rats at doses of 500, 750 or 1000 mg/kg as a single dose. In the results of the oral glucose tolerance test, it was reported that 3 hours after the administration of the 750 mg/kg dose, the blood sugar of diabetic rats decreased by 36.5%, while the blood sugar of normal rats decreased by 41.3%.

In another study, in which the antidiabetic effect of endophytic fungi isolated from medicinal plants was investigated, 66 cultures were tested against α-glucosidase inhibitors (AGI). Among these cultures, it was found that the majority of them belong to *Alternaria* and *Aspergillus* sp. and that *VRJ3* endophyte has the highest antidiabetic activity with a Decency of 91.57%. At the same time, they also emphasized that the AGI activities of these endophytic fungal species were tested for the first time in the study (Kaur *et al*., 2018).

Type 2, one of the most effective methods of treating diabetes is to inhibit the enzyme α-glucosidase, preventing the breakdown of complex carbohydrates and disabilities into monosaccharides, which are absorbed by the independent. Stojkovic and others (2019) analyzed the in vitro antidiabetic activities of medicinal mushroom species *C. militaris, C. comatus*, *Agaricus blazei*, *Inonotus obliquus*, *P. linteus* and *Morchella conica*. In the results, they emphasized that methanol extracts of *M. conica* and *C. militaris* did not inhibit the activity of α-amylase enzyme and that the species that inhibited α-glucosidase enzyme the strongest was

I. obliquus. At the same time, researchers have shown that *C. comatus* has a strong effect on the inhibition of the α-amylase enzyme.

Endophytic fungi are able to prevent diabetes by inhibiting the activity of α-amylase. Khan *et al*. (2019) aimed to determine the antidiabetic activities of these fungal species by isolating endophytic fungi from the leaves of different plant species in their studies. As a result of the study, it was reported that 8 endophytic fungi obtained with a 62% inhibition of amylase may be a potential source of antidiabetic drugs.

Vincent *et al*. (2013) investigated the changes in the group of animals fed a high-fat diet by using *A.blazei* mushroom for treatment in their studies. In the results, they reported that mushroom extracts prevent the body mass of living beings from increasing and the formation of new fat masses. In addition, they also emphasized that it protects against inflammation, glucose October intolerance and insulin resistance that occur as a result of a high-fat diet.

In a different study, researchers from the leaves of *Combretum dichoropetalum* the plant crude extracts and to isolate both oryzae nigrospor endophytic which is a type of fungi obtained from compound 3(S) (+) -2-cis-4-trans-abscisic acid (1), 7'-hydroxy-abscisic acid (2) and 4-des-hydroxyl altersolanol a(3)) antidiabetic effects assessed. The evaluation was carried out by monitoring the fasting blood sugar of alloxan-induced mice for a period of 9 hours. As a result of the study, they reported that both raw extracts and biosynthetic compounds of the endophytic fungus *Nigrospora oryzae* species have antidiabetic activity (Uzor *et al*., 2017).

Balaji *et al*. (2020) aimed to investigate the antidiabetic effect of the fungal species by using hot water and acetone extracts of *P. pulmonarius* micelles in diabetic albino rats. In the results, they stated that hot water extracts had a higher antidiabetic effect than acetone extract and that hot water extract was protective against diabetes-related complications by significantly lowering glucose.

In a different study with *C. militaris*, the antidiabetic effects of mushroom extracts on streptozotocin-induced diabetic mice were investigated. Within the scope of the study, the researchers used 0.05 g/kg and 2 g/kg aqueous or alcoholic extracts of *C. militaris* orally as a treatment method for diabetic mice for 3 weeks. In the results, they reported that the fungal species has a very strong antidiabetic activity (Dong *et al*., 2014).

In another study, the researchers aimed to evaluate the antidiabetic activities of the *P. florida* fungus and used methanol extracts for this. In the results obtained, it was reported that *P. florida* methanol extracts showed antidiabetic activity at 0.25, 0.50 and 1 mg α-amylase concentrations of 13.78%, 20.7% and 26.5%, respectively (Pandimeena *et al*., 2015). Khatun *et al*. (2013), on the other hand, gave dried powder extracts of *P. florida* fungus to diabetic rats and found that there was a decrease in blood sugar and cholesterol levels of the creature. It has also been reported that the LDH levels of rats given *P. florida* powder were low compared to the control group.

Kim *et al*. (2010) blood sugar level in normal rats 200-210 mg/dL, while streptozotocin-induced rats on the day the blood sugar level of the sixth and twelfth day, respectively 346 mg/dL 424 mg/dL is in the value of, and rats 30 mg/kg of *P. linteus* polysaccharide provision, reported that did not affect the decrease in blood sugar levels. However, significant lymphocyte infiltration was determined in many of the islet cells obtained from the control group, and it was reported that infiltration decreased in the group given polysaccharides, in contrast to the control group. In addition, when looking at the average insulin resistance indices, it was observed that in the control group it was 3.1 ± 0.4, while in the polysaccharide-treated group it was 0.69 ± 0.04.

1.3. Phytochemical Studies

Mollaei *et al*. (2019) aimed to determine the antioxidant potential of methanol extracts of endophytic fungi isolated from the roots of the *Lithospermum officinale* L. plant by polyphenol content and the presence of chiconin by HPLC in their study. The researchers reported that the species with the highest antioxidant activity in the results were the fungal species *Fusarium tricinctum* and *Alternaria altenata*, while the presence of chicory was detected only in the *F. tricinctum* species.

In another study, researchers aimed to make species diagnosis and perform phytochemical analysis by isolating endophytic fungi from the stems of the *Phragmanthera capitata* (Spreng.) Balle (Loranthaceae) plant. The species diagnosis was made macroscopically and microscopically, and in the results it was reported that 11 species belonging to 6 genera (*Aspergillus* 6 species, *Penicillium* 3 species, *Trichoderma* 1 species and *Fusarium* 1 species) were isolated. The phytochemical analysis results of these endophytic fungi revealed the presence of flavonoids, anthraquinones, tannins, phenols, steroids, coumarins and terpenoids in all of the extracts, while the absence of alkaloids and saponins was found. According to the data obtained, the researchers emphasized that the bioactive compounds of endophytic fungi isolated from the *P.capitata* plant are quite suitable for their use in agriculture and health fields (Ladoh-Yemeda *et al*., 2015).

Devi *et al*. (2012) Isolated the endophytic fungus *Penicillium* sp. from the *Centella asiatica* (L.) Urban plant and aimed to determine the contents of ethyl acetate extracts of the fungus by high-performance thin-layer Chromatography and HPTLC methods. In the study, the presence of phytochemicals such as phenols and flavonoids was first estimated quantitatively and then supplemented with high-performance thin-layer chromatography. The presence of alkaloids, phenols, flavonoids, tannins and glycosides in the analysis results was confirmed by the researchers and it was found that the raw extracts contained 2.76g of phenols and 0.275 g of flavonoids. With the results of HPTLC, it was reported that the presence of alkaloids and flavonoids was qualitatively clarified.

Mani *et al*. (2018) aimed to perform phytochemical analysis of dominant endophytic fungi of *Aegle marmelos* (*Curvularia australiensis* (FC2AP) and *Alternaria citri macularis* (FC8ABr)), which are found in many forests in India and are known to be a medicinal plant, in their study. In the results, it was found that these two endophytic fungi belonging to the same group contain flavonoids and phenols. They emphasized that fungal strains may have an important role in the use of the host plant as a medicinal plant due to the very high antioxidant activity due to the phenols and flavonoids they contain.

2. CONCLUSION

The consumption of mushrooms can offer nutritional and medicinal contributions to the human organism, especially due to their antioxidant activity. It is a fact that it is used as a raw material for many drugs used in the treatment of diseases such as cancer, Alzheimer's, and diabetes. Information about the importance and medicinal benefits of mushrooms was found in Ancient Egyptian hieroglyphs, in Ancient Greek and Roman texts, in Chinese medicinal texts. It can also be nominated to be a natural and reliable source of bioactive substances for applications in the pharmaceutical and cosmetic industries. In addition, more research is needed to develop new functional products and obtain other bioactive compounds from fungi.

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The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

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